Chlamydia pneumoniae infection of Dendritic cells and its role in asthma exacerbations

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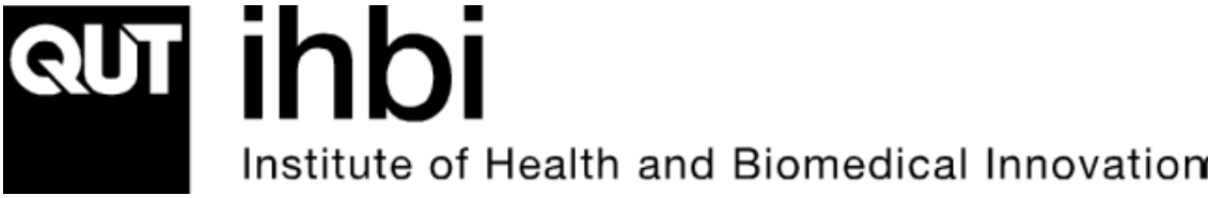

Abstract

Asthma is a common chronic inflammatory condition of the airways characterized by recurrent episodes of airway obstruction and wheezing. The causes of asthma are unclear but are known to include a mixture of genetic and environmental factors. Respiratory infections often interfere with the immune system which contributes significantly to asthma and this includes *Chlamydia pneumoniae*. *C. pneumoniae* is a ubiquitous, obligate, intracellular, Gram-negative bacterium that commonly causes acute respiratory illness. In recent years, accumulating evidence from sero-epidemiological studies has shown that many asthmatics have elevated antibody levels to *C. pneumoniae*, suggestive of persistent infection but causal evidence is lacking.

DCs (Dendritic cells) are shown to be involved in *Chlamydia* induced asthma. Studies from murine models have shown that chlamydial infection of DC *in vitro* can demonstrate distinct inflammatory responses which are skewed towards a Th2 phenotype. Furthermore, the injection of *Chlamydia*-infected DCs into the airways of naive mice induced all features of asthma which demonstrates the *in vivo* capacity of *Chlamydia*-infected DCs to initiate allergic airway inflammation. Hence, this unique and potent Th2 adjuvant characteristics of *Chlamydia* which are partially mediated through DCs, may explain the mechanism behind this strong association between *Chlamydia* and asthma. DCs are the most important antigen-presenting cells in the lung and are mainly recognized for their exceptional potential to generate a primary immune response to optimally clear infectious threats. However, DCs can also mount maladaptive Th2 immune responses to harmless allergens and respiratory pathogens which contribute to immunopathology in asthma.The work presented in this thesis examined the interaction of *C. pneumoniae* with monocyte-derived DCs (MoDCs) from both asthmatic patients and healthy controls. We investigated the comparative effect of *C. pneumoniae* infection on MoDC maturation markers from healthy controls, acute asthma and stable asthma cohorts along with addressing differences in cytokine secretion profile. Our analyses revealed that *C. pneumoniae* infection in MoDCs induces a significant down-regulation of CD80 marker in MoDC from both healthy controls and asthma cohorts. In terms of differences, only *C. pneumoniae*-infected MoDCs from the stable asthma cohort displayed a significant up-regulation of CD83. Cytokine analysis revealed that the stable *C. pneumoniae* infection of MoDCs from stable asthma patients leads to a non-significant trend towards increase in production of cytokines IL-1β, IL-6, IL-8 and TNF-α.

To gain further insight into role of *C. pneumoniae* in asthma, microarray analysis was used as a screening tool to compare transcriptional profiles of *C. pneumoniae*-infected MoDCs from asthma cohorts and healthy controls. A number of genes and gene families were specifically up-regulated in the *C. pneumoniae*-infected MoDCs from the asthma cohorts that can facilitate asthma exacerbations.

Hence, this study has demonstrated that exposure to infectious *C. pneumoniae* can have an effect on asthma through expression of airway remodelling genes on DCs and generation of inflammatory responses. This provides a more comprehensive understanding of the magnitude of the impact of *C. pneumoniae* infections on asthma development which could establish the groundwork for new therapeutic strategies to inhibit the development of asthma.

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List of Abbreviations

ADAM	A disintegrin and metallopeptidase
AHR	airway hyperresponsiveness
APC	Antigen Presenting Cell
ASM	airway smooth muscle
BAL	Bronchoalveolar lavage
BALF	Broncho alveolar lavage fluid
BEAS-2B	Human bronchial epithelial cells
BEC	bronchial epithelial cell
CCL	Chemokine ligand
CD	Cluster of differentiation
CLSM	Confocal laser scanning microscopy
Cp-HSP60	*C. pneumoniae* 60 kD heat shock prot
CCR7	C-C chemokine receptor type 7
cysLTs	cysteinyl leukotrienes
DCs	Dendritic cells
Df/Derf	Dermatophagiodes farinae
DMEM	Dulbecco's Modified Essential Medium
DNA	Deoxyribo nucleic acid
EB	Elementary bodies
ECP	eosinophil cationic protein
EDN	eosinophil derived neurotoxin
FCS	Fetal calf serum
FEV1	Forced Expiratory Volume at 1 second
GATA3	GATA binding protein 3
GM-CSF	Granulocyte macrophage colony stimulating factor
hCLCA1	human calcium-activated chloride channel
Hep-2	Human Epithelial
HLA-DR	Human Leukocyte antigen class 2
HSP70	Heat Shock Protein 70
IDO	Indoleamine-2, 3- dioxygenase
IFNγ	Interferon gamma
IFU	Inclusion forming unit
IgE	Immunoglobulin E
IL-4R	Interleukin 4 receptor
IL	Interleukin
iTreg	inducible regulatory T cell
JAK	janus-tyrosine kinases
LTC_4	leukotriene C_4
MBP	Major basic protein
M-CSF	Macrophage colony stimulating factor
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
NF-κB	Nuclear factor kappa B

OVA	ovalbumin
PAF	platelet activating factor
PAI-1	Plasminogen activator inhibitor-1
PBMC	Peripheral blood mononuclear cells
PCH	Prince Charles Hospital
PCR	Polymerase chain reaction
PGE	prostaglandin E
PRR	pattern recognition receptors
RB	reticulate body
rRNA	ribosomal Ribonucleic acid
SEM	Standard error of the means
SERPINB2	Serpin peptidase inhibitor member 2
TCR	T cell receptor
TGFβ	transforming growth factor beta
Th	T helper
TLR	Toll like receptor
TNF-α	Tumor necrosis factor alpha
Treg	T regulatory cells
TXO2	thromboxane A2
VCAM-1	Vascular cell adhesion molecule 1
VEGF-A	Vascular Endothelial Growth factor A
ZO-1	Zona occludens-1

Declaration

The work contained in this thesis has not been previously submitted to meet requirements for an award at this or any other higher education institute. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person, except where due reference is made.

QUT VERIFIED SIGNATURE 14 / 04 / 2014
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Chapter One: Introduction and Literature review

Background

Asthma is a chronic obstructive disease of the airways, characterized by an inappropriate immune response that results in bronchoconstriction, mucus secretion, and eosinophilic or neutrophilic airway inflammation. All of these features of asthma compromise an individual's capacity to maintain normal respiratory functions, particularly when exposed to ubiquitous airborne environmental stimuli and respiratory pathogens. Around 300 million people worldwide are known to be affected by asthma and it is projected that this number would increase to 400 million by 2025, as countries become more urbanized (Masoli et al., 2004; To et al., 2012). Asthma is Australia's most widespread chronic (long-term and persistent) health problem and affects over 10% of the Australian population (Pawankar et al., 2012). The economic cost associated with asthma in Australia is estimated to be around $354 million per year and this includes direct costs associated with hospital visits, asthma medications, outpatient clinic costs of physician visits and other indirect costs of asthma relating to lost work days (Bahadori et al., 2009).

A clinical history in combination with a reversible airway obstruction as measured by a pulmonary function test is the gold standard for diagnosing asthma (To et al., 2012). Pulmonary function tests (particularly the Forced Expiratory Volume in the first second (FEV_1) measurement from spirometry) and peak expiratory flow (PEF) measurement can help clinicians to assess the severity of airflow limitation and document response to therapy. These measurements can help diagnose asthma in patients who have poor perception of their symptoms (Pruitt and Lawson, 2011). Asthma is estimated to account for 1 in every 250 deaths worldwide, resulting in 250,000 deaths each year worldwide (To et al., 2012).

The immune system must recognize pathogenic stimuli and respond appropriately, as immune responses are strictly regulated to protect the host from exaggerated signals that may cause tissue injury by establishing a state of immune tolerance. Certain environmental factors, such as exposure to allergens, and genetic factors disturb this fine balance of immune regulation, leading to allergic disorders such as asthma (Soyer et al., 2012). Although allergens are ubiquitously distributed in the environment, the normal response to allergens in healthy individuals is either absent or results in antigen-specific immune tolerance. Under non-allergic conditions, soluble proteins do not induce a vigorous immune response.

An interesting aspect of asthma is its heterogeneous, complex nature and the fact that it can present as a chronic, stable disease and as asthma exacerbations (Edwards et al., 2012). Asthma is thought to develop in two stages (Moore et al., 2010). The first stage, known as sensitization, encompasses the exposure to a normally innocuous antigen in the lungs during some type of inflammatory response that leads to the development of Th2 type memory cells (Locksley, 2010). Sensitization to a specific antigen is a prerequisite for the onset of atopic diseases in predisposed individuals, which is dependent on the potential of the allergen to prime the CD4+ T helper type 2 (Th2) cell response in which interleukin-4 (IL-4) and IL-13 drive immunoglobulin E (IgE) class-switch in B cells (Soyer et al., 2012). Later, upon re-exposure to the same antigen, these memory cells are activated, resulting in an inflammation of the lungs. There are many possible mechanisms for antigen sensitization to occur, and one increasingly important scenario involves respiratory infections (Sly et al., 2010). Sensitization refers to the ability of the allergen to elicit a Th2-cell response leading to IgE production (Galli and Tsai, 2012). Allergic sensitization which triggers asthma can present at any age, but its highest prevalence is during childhood and young adulthood. An interaction between early

exposure to allergens, respiratory infections and family history are probably important in the initiation of allergic asthma, but the mechanisms by which some children develop asthma, whereas others have non-respiratory allergic manifestations, are not understood (Ma and O'Byrne, 2012).

Factors that influence asthma development include host and/or environmental factors (Pruitt and Lawson, 2011). Host factors that increase the risk of asthma include genetic predisposition, obesity, and gender while environmental factors that increase the risk of asthma include allergens, air pollution, respiratory infections and diet (Pruitt and Lawson, 2011). Allergen challenge in patients with asthma provokes the influx of activated Th2 cells into the airways and this is accompanied by an increase in the secretion levels of Th2-type cytokines, such as IL-4, IL-5, IL-9 and IL-13, which are known to drive the pathology seen in asthma. An allergen is defined as a non-pathogenic environmental antigen that elicits inappropriate immune responses (Asokananthan et al., 2002). Respiratory infections can also play a similar role in the development of asthma as they are known to trigger asthma exacerbations in both adults and children which includes both viral and bacterial infections (Jackson et al., 2011).

Host factors

Genetics: Asthma has a heritable component with current data showing that genetic factors undoubtedly contribute to asthma susceptibility (Binia and Kabesch, 2012; Slager et al., 2012). The search for genes linked to development of asthma has focused on three major areas: production of allergen-specific IgE antibodies (atopy); expression of airway hyperresponsiveness; and generation of inflammatory mediators such as cytokines, chemokines & growth factors (Slager et al., 2012). A genome-wide study for genetic factors associated with total serum IgE levels in 7087 subjects with asthma and 7667 controls, has identified one novel locus in the MHC class II region which is significant at the genomewide level (Moffatt et al., 2010). Genes associated with expression of airway hyperresponsiveness are put down to LTC_4 synthase, encoded on chromosome 5q35, and necessary for synthesis of cysteinyl-leukotrienes, LTC_4. The cysteinyl leukotrienes LTC_4, LTD_4, and LTE_4 are critical mediators of airway narrowing, microvascular leakage, mucus secretion, and eosinophilia in bronchial asthma (Slager et al., 2012). Studies have shown that in patients with aspirin-intolerant asthma (AIA) the expression of LTC_4 in mast cells and eosinophils is increased 5-fold in parallel with enhanced Cyst-LT production (Cowburn et al., 1998). The chromosome region 5q31–33 is often seen as a major susceptibility locus for asthma as genes encoding for Th2-type cytokines cytokines are located in this region and activation of Th2 cells leads to increased transcription of these genes which eventually leads to increased cytokine secretion from activated Th2 cells (Renauld, 2001).

Environmental factors

Allergens: An allergen is an antigen capable of stimulating a type-I hypersensitivity reaction in atopic individuals through IgE responses. A large body of evidence from epidemiological studies has demonstrated that IgE–mediated sensitization to inhalant allergens is an important risk factor for asthma particularly in childhood. Sensitization to an allergen reflects the allergen's ability to elicit a Th2-cell response, in which IL-4 and IL-13 drive IgE production by promoting immunoglobulin class-switch recombination in B cells (Galli et al., 2008). Many factors affect the likelihood of developing clinically significant sensitization: host genotype, type of allergen, allergen concentration

in the environment and whether exposure occurs together with agents that can enhance the sensitization process (Galli et al., 2008). The early allergic responses are mediated primarily by IgE-dependent processes. These immune responses are initiated when IgE binds to high-affinity receptors (FcεR1) located on the surfaces of mast cells and basophils and is subsequently cross-linked by allergen, causing cell activation with the release of inflammatory mediators. The role of indoor allergens, such as house dust mite (HDM), furry pets, and also fungi, in the development of allergy is widely implicated (Jayaratnam et al., 2005). Positive skin prick test responses to allergens at baseline were the most important determinant for both the incidence and persistence of allergic asthma (Jayaratnam et al., 2005). Atopy is often identified as the strongest predisposing factor for the development of asthma (Holt and Sly, 2011) as the titers of allergen-specific IgE in serum are often useful in predicting persistent wheeze and in targeting allergen specificities for allergen avoidance management.

Air pollution: Epidemiological and clinical research continues to support a link between urban air pollution, the pre- and postnatal exposure to tobacco smoke and an increased incidence of airway disease (Baiz and Annesi-Maesano, 2012). The most consistent finding for asthma induction in childhood is related to exposure of environmental tobacco smoke. Building upon previous findings from 79 prospective studies, a recent meta-analysis showed that exposure to passive smoking increases the incidence of wheeze and asthma in children and young people by at least 20% and that preventing parental smoking is crucially important to the prevention of asthma (Burke et al., 2012). Maternal smoking during pregnancy is associated with increased risks of wheezing and asthma during childhood (Duijts, 2012) with a study demonstrating that maternal smoking affects fetal lung growth in the second and third trimesters of pregnancy which leads to delivery of smaller infants who go on to have adverse respiratory outcomes in childhood (Prabhu et al., 2010).

Diet: The rise of asthma prevalence during the last decade among children in developed countries as well as developing countries have led investigators to link the allergic respiratory disease to dietary factors. Amongst dietary factors breast-feeding has been particularly implicated as data reveal that infants fed formulas of intact cow's milk or soy milk have a higher incidence of wheezing illnesses in early childhood compared with those fed breast milk (Bener et al., 2007). Other dietary factors proposed to explain the rise in asthma prevalence include decreased intake of fruits/vegetables and increased intake of "Westernized" processed foods (Varraso, 2012).

Respiratory infections: A new paradigm for asthma is developing in which infection is seen as a risk factor for the development and the propagation of asthma. Both viruses (mainly rhinovirus, respiratory syncytial virus (RSV) and influenza A) and bacteria (*Mycoplasm. pneumoniae* and *Chlamydia pneumoniae*) are known to play a role in this paradigm (Martin, 2006). Studies have demonstrated that children hospitalized for RSV bronchiolitis during infancy are more likely to have subsequent episodes of wheezing and asthma during the first decade of life compared with children who didn't have a history of a bronchiolitis hospitalization during infancy (Henderson et al., 2005). Moreover, several epidemiologic studies suggest that infants who develop severe viral respiratory infections are more likely to have asthma later in childhood. This evidence is strongest for respiratory infections caused by respiratory syncytial virus (RSV) and rhinoviruses (RVs). However, the link between respiratory viral infections and asthma development remains unclear (Caramori et al., 2012; Dakhama et al., 2005). The four main causative mechanisms hypothesized in the

association between viral respiratory infections and the subsequent development of asthma in children are (1) alterations in airway function and size; (2) dysregulation (congenital and acquired) of airway tone, (3) alterations in the immune response to infections; and (4) the host genetic variants involved in immune response(Caramori et al., 2012; Dakhama et al., 2005).

There is an increasing body of literature concerning the association between the atypical intracellular bacteria *Chlamydia pneumoniae* and *Mycoplasma pneumoniae* and asthma pathogenesis. The atypical bacteria *Mycoplasma pneumoniae* and *Chlamydia pneumoniae* are common respiratory pathogens and have also been associated with asthma, wheeze and asthma exacerbations in both adults and children (Edwards et al., 2012; Johnston and Martin, 2005). Detection rates for these bacteria can be as high as 80% in patients with these conditions, and serological positivity rates for these atypical bacteria are often 40–60% (Edwards et al., 2012). However, many studies investigating such a link have been uncontrolled and have provided conflicting evidence, in part because of the difficulty in accurately diagnosing infection with these atypical pathogens (Caramori et al., 2012). Biscardi and colleagues (Biscardi et al., 2004) found that *M. pneumoniae* was a causative microbe in 20% of exacerbations in asthmatic children requiring hospitalization for asthma exacerbations. This high rate was not confined to previously diagnosed asthmatics. Fifty percent of children experiencing their first asthmatic attack were also positive for *M. pneumoniae*. These figures were significantly greater than for other bacteria or viruses that were evaluated in the study which led to suggestions that *M. pneumoniae* seems to play an important role both in initial and subsequent asthma exacerbations (Martin, 2006). Thereby, respiratory infections are shown to cause wheezing episodes in children and can influence the onset and severity of asthma via complex and intersecting mechanisms.

Hygiene hypothesis and asthma

The hygiene hypothesis attempts to provide a biological explanation for epidemiologic observations of decreased incidence of allergic disease in households with large numbers of siblings. The concept of the hygiene hypothesis was first introduced by Strachan in 1989 (Strachan, 1989), based on the observation that there was an inverse correlation between household size and allergic rhinitis. Strachan (Strachan, 1989) postulated that the incidence of infection was higher in households with more family members and that this increased incidence of infection reduced the subsequent development of allergic rhinitis. Strachan's hypothesis, which originated from epidemiological studies only, was given a biological explanation as investigators in recent years have noted the lower prevalence of asthma and atopy among children raised on a farm (von Mutius and Radon, 2008). The study by Ege and colleagues (Ege et al., 2011) compared children living on farms with those in a reference group with respect to the prevalence of asthma and atopy, and to the diversity of microbial exposure, in two cross-sectional studies involving more than 16,500 elementary school children. The study (Ege et al., 2011) investigated exposure to indoor dust using different methods and findings indicate that indoor microbial exposure is much more common and diverse in the farming environment than in the non-farming environment of the reference group. The authors (Ege et al., 2011) speculated that combinations of microbial exposures may activate several signaling pathways downstream of these receptors, with subsequent induction of regulatory T cells.

A complicated story is also emerging as subsequent studies have emphasized the critical importance of immune maturation in the development of the allergic phenotype. The potential role of microbial burden on the development of allergic phenotype during this initial period of immune maturation has gained considerable epidemiologic and experimental support. Studies (Prescott et al., 1999; Upham et al., 2002; Yerkovich et al., 2007) have shown that the impaired capacity to induce Th1 immune responses that normally occurs over the first 12 to 18 months of life is what predisposes to the subsequent development of allergy and asthma. Moreover, several epidemiologic studies suggest that infants who develop severe viral respiratory infections are more likely to have asthma later in childhood (Ahanchian et al., 2012; Henderson et al., 2005). Thus, the nature of respiratory infections and normal maturation of the immune system is postulated to drive the development of asthma.

So far a unifying concept is still lacking, hence the hygiene hypothesis is not likely to be the sole explanation for the ongoing asthma epidemic in industrialized nations.

Pathology in asthma or Hallmark features of asthma

Mucus overproduction

Mucus is a liquid gel that lines the inner surface of the airways and forms a first line of innate defense against inhaled insult. It protects the underlying airway epithelium from harm by trapping and promoting the removal of foreign debris. Mucus is produced by submucosal glands and goblet cells scattered along the airway epithelium (Lai and Rogers, 2010). Goblet cells have electron dense granules containing mucin, the main component of mucus. Mucins consist of a peptide backbone, to which multiple oligosaccharide side chains are bound, with carbohydrates accounting for 70–80% of the total mass of the mucin molecule (Lai and Rogers, 2010). The complete mucin glycoprotein, which is highly sulphated, is accumulated in secretory granules and is released following appropriate stimulation by aeroallergens (Renauld, 2001). Mucins provide mucus with viscoelasticity and constitute up to 2% by mass. Mucins are encoded by a specific group of mucin (MUC) genes and examples of airway mucins include MUC2, MUC4, MUC5AC and MUC5B (Lai and Rogers, 2010). Airway goblet cells primarily express MUC5AC while MUC5B is predominantly expressed by mucus cells in submucosal glands. Both MUC5AC and MUC5B can be up-regulated by inflammatory mediators such as Th2 cytokines (Lai and Rogers, 2010).

Mucus hypersecretion during asthma can become pathological as mucus accumulation obstructs the airways, causing respiratory distress and sometimes mortality. Marked goblet cell hyperplasia with accumulation of mucus has been reported in the airway of patients who died from severe acute asthma attack (Aikawa et al., 1992). Excessive mucus production during asthma is mediated by Th2 cytokines, IL-9 and IL-13, with IL-13 being the crucial mediator in mucus secretion. IL-13 binds to IL-4Rα which then leads to phosphorylation of signal transducer and activator of transcription 6 (STAT6) with down-regulation of forkhead box A2 (FOXA2). This then leads to mucin gene expression, with consequent increases in mucin synthesis (Lai and Rogers, 2010). IL-9 may contribute to mucus hypersecretion in asthma by up-regulating the human calcium-activated chloride channel, hCLCA1. hCLCA1 encodes a calcium activating chloride channel which controls the flux of chloride (Cl^-) ions across the epithelium (Wills-Karp, 2004). Thus, hCLCA1 may be partly responsible for mucus

overproduction in asthma, as well as regulating mucus volume by mediating chloride and water outflow. Mucus hydration may increase viscosity by facilitating osmotic movement of plasma exudates into the airway lumen.

Besides directly driving mucin gene expression, IL-13 is also indirectly responsible for mucus overproduction(Lai and Rogers, 2010). One such pathway is the TNF-α signaling pathway. TNF-α is released by both Th2 cells and neutrophils when neutrophils are recruited to the airways by an IL-8-like chemoattractant synthesized by airway epithelial cells in response to IL-13 stimulation (Lai and Rogers, 2010). TNF-α mediated mucus production is dependent on ligation of TNF-α with its receptor leading to activation of the transcription factor nuclear factor NF-κB, which eventually drives the expression of MUC genes (Lai and Rogers, 2010).

Eosinophil infiltration

Eosinophils are multifunctional leukocytes which account for a small percentage of peripheral-blood leukocytes, and their presence in tissues is primarily limited to the gastrointestinal mucosa (Rothenberg, 1998; Rothenberg and Hogan, 2006). They are produced in the bone marrow from pluripotent stem cells which then differentiate into CD34+ progenitor cells (Rothenberg and Hogan, 2006; Walsh and August, 2010). Eosinophils then develop from the CD34+ progenitors under the influence of IL-5, which can be produced by CD4+ Th2 cells, basophils, mast cells and CD8+ T cells. Following development, eosinophils then migrate into the bloodstream, circulate through the lymphoid tissue and commonly home to the gut and uterus (Rothenberg and Hogan, 2006).

Eosinophil infiltration of the bronchial mucosa is a feature of the airways of patients who have died of asthma and is present in bronchial biopsy specimens and bronchoalveolar lavage (BAL) of patients with asthma (Kroegel, 1990). The increased number of eosinophils count in the blood is termed eosinophilia. During an allergic asthma response, eosinophils are recruited to the lungs by means of chemokines and cytokines. Trafficking of eosinophils from the gut to the lungs is first initiated through secretion of chemokines such as CC-chemokine ligand 11 (CCL11) (also known as eotaxin-1) and CCL24 (also known as eotaxin-2) which are produced by the airway epithelium (Walsh and August, 2010). Once eosinophils reach the lungs, they infiltrate the site of inflammation through interacting with the blood vessels surrounding the bronchial mucosa. Eosinophil infiltration into lungs are aided by Th2 cells through secretion of IL-5 which induces changes in blood vessels that lead to the up-regulation of intracellular adhesion molecule 1 (ICAM1) and vascular cell-adhesion molecule 1 (VCAM1). This allows the infiltration of very late antigen 4 (VLA4)-expressing eosinophils into the airway wall which eventually mediates the damage in the airway epithelium (Rothenberg and Hogan, 2006). Secretion of cytokines such as IL-3, IL-5 and GM-CSF by Th2 cells in the lungs ensures the activation and survival of eosinophils for extended period of time. In the absence of these cytokines, eosinophils undergo apoptosis in less than 48 hours with rapid clearance by macrophages (Rothenberg, 1998). Eosinophils mediate their immune responses through secretion of toxic inflammatory molecules (Figure 1.1) which are synthesized after cellular activation and are stored in granules. These granules in eosinophils are selectively released through three different mechanisms (Figure 1.1) in response to diverse stimuli, including nonspecific tissue injury, infections, allografts, allergens, and tumors (Kroegel, 1990):

i) Nonoxidative mechanism: This is mainly mediated by three different cationic proteins stored in the specific eosinophil granules: Major basic protein (MBP), eosinophil derived neurotoxin (EDN) and

eosinophil cationic protein (ECP) (Figure 1.1). ECP can cause voltage-insensitive, ion-nonselective toxic pores in the membranes of respiratory epithelium and these pores may facilitate the entry of other toxic molecules (Rothenberg, 1998). MBP directly increases smooth muscle contraction responses by dysregulating vagal muscarinic M2 and M3 receptor function. MBP also induces mast cell and basophil degranulation, which causes release of histamine molecules. Elevated levels of eosinophil granule proteins (e.g., MBP) have been found in bronchoalveolar lavage fluid from patients with asthma, and importantly these concentrations are sufficient to induce cytotoxicity of a variety of host tissue, including respiratory epithelial cells *in vitro*. EDN is a ribonuclease which is shown to possess antiviral activity (Rothenberg and Hogan, 2006).

ii) Oxidative mechanism: involves generation of highly reactive oxygen species by eosinophil peroxidase (EPO) (Figure 1.1). EPO generates these reactive oxygen species using the respiratory-burst–oxidase pathway in eosinophils (Rothenberg, 1998). These reactive oxygen molecules oxidize nucleophilic targets on proteins, thereby promoting oxidative stress and subsequent cell death by apoptosis and necrosis. The target molecules can be located either in the cell or invading microorganisms.

iii) Humoral mechanism: involves de novo generation and release of lipid mediators such as platelet activating factor (PAF), leukotriene C_4 (LTC_4), prostaglandin E (PGE) and thromboxane A_2 (TXO_2) (Figure 1.1). Human bronchial smooth muscles are extremely sensitive to these lipid mediators which cause bronchoconstriction in late phase asthmatic reactions. Leukotriene C_4 (LTC_4) is metabolized to leukotriene D4 and leukotriene E4. All the leukotrienes are slow-reacting substances of anaphylaxis that increase vascular permeability and mucus secretion and are potent stimulators of smooth-muscle contraction (Rothenberg, 1998).

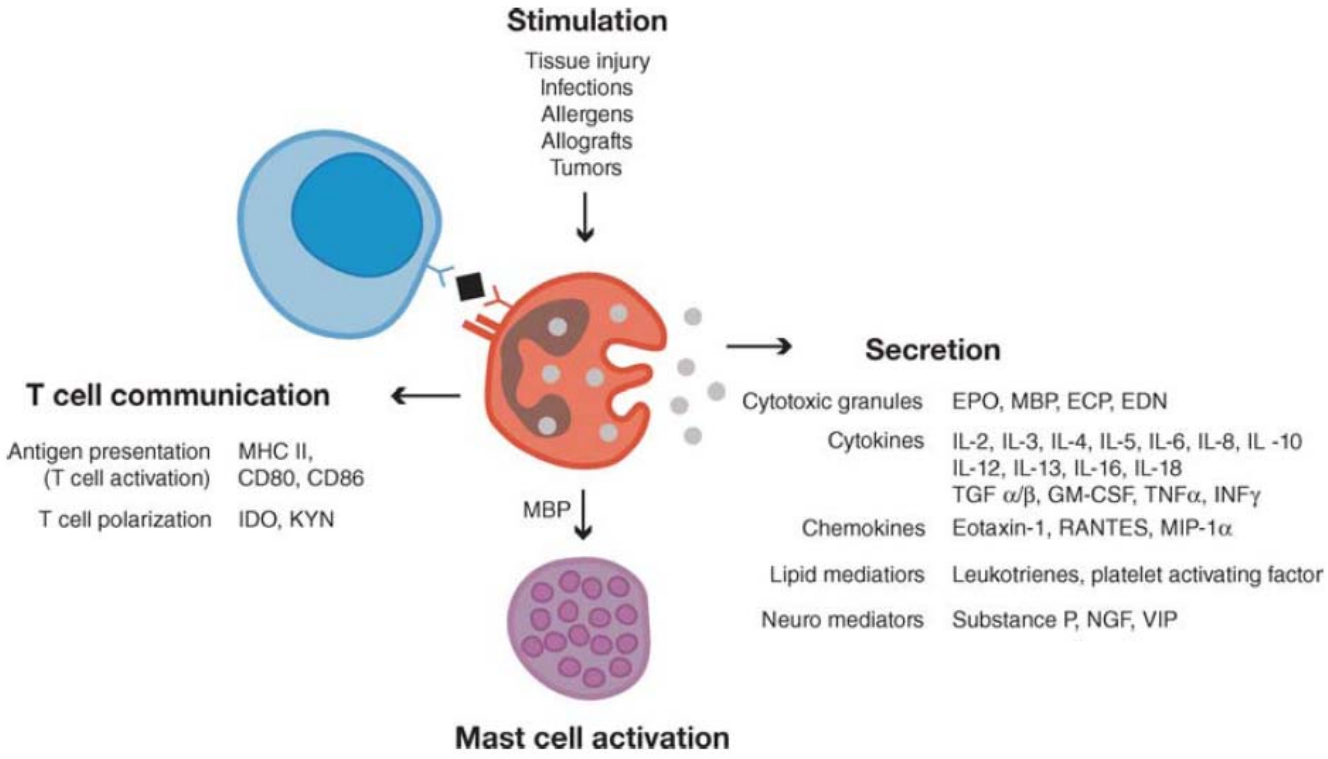

Figure 1.1: Multifunctional effects of the eosinophil in the immune system. Eosinophils are bilobed granulocytes with eosinophilic staining secondary granules. The secondary granules contain four primary cationic proteins, designated eosinophil peroxidase (EPO), major basic protein (MBP), eosinophil cationic protein (ECP), and eosinophil-derived neurotoxin (EDN). All four proteins are cytotoxic molecules; in addition, ECP and EDN are ribonucleases. Eosinophils respond to diverse stimuli, including nonspecific tissue injury, infections, allografts, allergens, and tumors (Rothenberg and Hogan, 2006). In addition to releasing their preformed cationic proteins, eosinophils can also release a variety of cytokines, chemokines, lipid mediators, and neuromodulators. Eosinophils directly communicate with T cells and mast cells in a bidirectional manner. Eosinophils activate T cells by serving as Antigen-Presenting Cells, and can also regulate T cell polarization through synthesis of indoleamine 2,3-dioxygenase (IDO), an enzyme involved in oxidative metabolism of tryptophan, catalyzing the conversion of tryptophan to kynurenines (KYN), a regulator of Th1/Th2 balance. Figure taken from (Rothenberg and Hogan, 2006).

Airway hyper-responsiveness (AHR)

Airway hyperresponsiveness (AHR) is defined as an exaggerated bronchospastic response or bronchoconstriction of the airways caused in response to a provoking agent such as allergens (Wills-Karp et al., 1998; Woolcock and Peat, 2000; Zhu et al., 1999). The airway wall of patients with asthma is characterized by increased smooth muscle mass, mucous gland hypertrophy and vascular congestion leading to a thickened airway wall and markedly reduced airway diameter (Figure 1.2). These features leads to the development of airflow limitation by increasing airway resistance (Figure 1.2) (Saetta and Turato, 2001). Airway wall thickness is increased 50–300% in cases of fatal asthma and 10–100% in cases of nonfatal asthma , compared with non asthmatic controls (Elias et al., 1999). Contraction of the airway smooth muscle (ASM) is the principal component underlying this bronchoconstriction that characterizes the acute phase of an asthmatic attack. Action of Th2 cytokines on the ASM is thought to induce these changes on the ASM, thereby, initiating AHR (Shore, 2004).

One such mechanism is the action of IL-13 on the ASM. ASM has been shown to express receptors for IL-13, which includes IL-13Rα1 and IL-4Rα, and binding of IL-13 to IL-13Rα1 leads to dimerization of this receptor with IL-4Rα (Laporte et al., 2001). Upon dimerization of IL-13Rα1 with IL-4Rα, Janus-tyrosine kinases (JAK) constitutively associated with the receptors become phosphorylated and activated and subsequently phosphorylate tyrosine residues on the IL-4Rα and IL-13Rα1. This is followed by binding of STAT6 monomers to phosphorylated tyrosine residues of IL-4Rα. Once bound, STAT-6 becomes phosphorylated by JAKs, whereupon it is released from the receptor, dimerizes with other phosphorylated STAT-6 molecules, translocates to the nucleus, and induces gene transcription (Laporte et al., 2001). This then subsequently leads to expression of cysteinyl leukotriene receptor 1 (CysLT1) the receptor for LTD_4 which induces changes in stiffness of the ASM cells and thereby initiates bronchoconstriction of the airways (Shore, 2004). Binding of IL-13 to ASM also leads to transcription and release of chemokines (Peng et al., 2004) which ultimately attracts eosinophils to the airway wall leading to eosinophilia in the airway epithelium.

Increased proliferation of ASM has is also known to contribute to AHR as it has been shown that ASM cells in culture, obtained from asthmatic patients, grow at twice the rate of those from non asthmatic control individuals (Black and Johnson, 2002). The ASM lies within an extracellular matrix (ECM) that is intimately connected with its function. The ECM in the lung turns over at a rate of 10-15% per day. This turnover is a result of the actions of endogenous matrix metallo proteinases (MMPs), which digest the matrix, and their inhibitors (tissue inhibitors of MMPs, TIMPs). Some of the proteins which make up the ECM includes collagens I, III and V and fibronectin. Increased

deposition of the ECM proteins are observed in the airway biopsies from asthma patients which leads to bronchoconstrcition and impaired airway function (Black and Johnson, 2002). There are few data regarding the effect of Th2 cytokines on ASM proliferation (Shore, 2004).

Both the combined effects of increased proliferation of ASM and contractile responses of ASM lead to airway remodelling in asthmatic patients. Airway remodelling is a term that indicates the structural changes occurring in the airways due to change in the composition, quantity and organization of the cellular and molecular components of the airway wall. Other than epithelial damage, these include alterations in mucus-secreting structures, increase in smooth muscle mass, increased vascularity, matrix abnormalities and thickening of the airway wall (Zhu et al., 1999). Airway wall remodelling is a prominent feature of the asthmatic airway (Zhu et al., 1999). In conclusion, structural alterations generated in the airways of asthmatic patients are a consequence of chronic inflammation and contribute to the symptoms and physiological dysregulation seen in asthma.

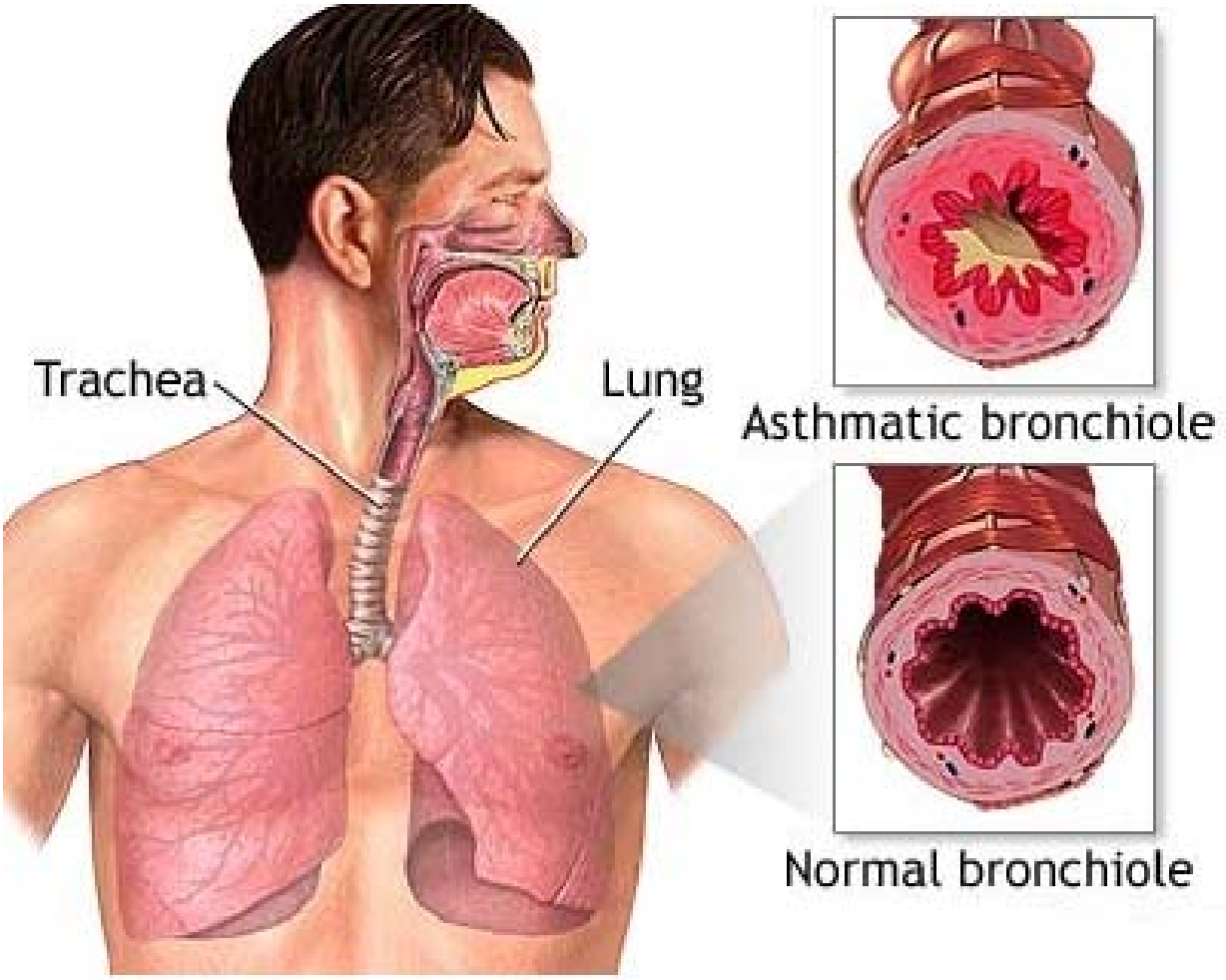

Figure 1.2: Representation of the bronchioles in the healthy and asthmatic patients. The bronchoconstriction of the airways, observed in asthma patients, is the dominant physiological symptom of asthma. Bronchoconstriction could be caused by many factors including mucus hypersecretion, contraction of the airway smooth muscle, stimuli (such as exercise, cold air and irritants) and allergen exposure in the lungs to Th2 cells which secrete IL-13. Figure taken from (www.world-drugs.net)

Basement membrane thickening

Histologically, reticular basement membrane thickening of airway epithelium is a characteristic feature of asthma (Figure 1.2) and it has been demonstrated to be directly related to disease severity (Niimi et al., 2000). The epithelial basement membrane consists of two layers: shallow layer (basal lamina) and deeper layer (lamina reticularis). When thickened basement membrane of asthmatic airway is observed with electron microscopy, a thickening of the area just below the lamina reticularis, is observed. The thickening of subepithelial space corresponds to the deposition of collagen I, III, V and extracellular matrix (ECM), such as fibronectin, laminin and tenascin (Tagaya and Tamaoki, 2007). The mechanism of deposition of ECM is thought to be an imbalance between its synthesis and degradation which is controlled by plasminogen activator inhibitors (PAIs) and metalloproteinases (MMPs). Furthermore, increased vascularity in the airway epithelium can contribute to reticular basement membrane thickening of airway epithelium contributing to bronchial constriction (Figure 1.2).

i) Plasminogen activator inhibitors (PAIs)

PAIs belong to the family of serine protease inhibitors and play a crucial role in various physiological processes, including fibrinolysis, ECM turnover, tissue repair, blood coagulation, thrombolysis, ovulation, embryogenesis, angiogenesis, and cell adhesion and migration through its interactions with plasminogen activators (PA), urokinase-type plasminogen activator (uPA) receptors, and vitronectin (Aso, 2007).

Plasminogen activator inhibitor-1 (PAI-1) is the main inhibitor of the fibrinolytic system and is known to play an essential role in airway remodelling. The fibrinolytic system plays a key role in ECM degradation in the airways. The fibrinolytic system comprises an inactive proenzyme, plasminogen, that can be converted to the active enzyme, plasmin by two physiological plasminogen activators (PA), the tissue type PA (t-PA) and the urokinase type PA (u-PA) (Ma et al., 2009). Plasmin directly degrades various types of ECM proteins including fibrin, laminin, proteoglycan, fibrin, and collagens. It can also activate MMPs, which in turn degrade ECM proteins (Ma et al., 2009).

An important mechanism in the regulation of PA activity is inhibition of uPA or tPA by three major inhibitors, which are PAI-1, PAI-2, and PAI-3 (Ma et al., 2009). PAI-3 regulates enzymes involved in fertilization rather than lung tissue fibrosis, whereas altered expression of both PAI-1 and PAI-2 is of potential relevance to the process of lung fibrosis. Although PAI-2 exhibits inhibitory activity toward tPA and uPA (26, 27), the efficiency of PAI-2 is 20- to 100-fold less than what is observed for PAI-1 (Ma et al., 2009)

Increased PAI-1 production has been observed in serum samples from asthma patients (Cho et al., 2011) and mast cells has been pointed as one of the main sources of PAI-1 production (Cho et al., 2000). In murine models of asthma, PAI-1 production promotes the deposition of ECM in the airways causing irreversible airway structural changes such as deposition of collagen and fibrin in the airways (Oh et al., 2002).

ii) Matrix metalloproteinases (MMPs)

MMPs are a family of zinc-dependent proteases that were initially identified by their ability to degrade collagen. MMPs form a group of more than 20 zinc-dependent enzymes that are involved in the remodelling of several components of the extracellular matrix (ECM) (Hu et al., 2007). They play a role in many physiological processes, such as embryo implantation, bone remodelling and organogenesis, and have additional roles in the reorganization of tissues during pathological conditions such as airway inflammation, wound healing and invasion of cancer cells. MMPs can be broadly classified on the basis of substrate specificity into collagenases (MMP-1, 8, 13, 18 and 19), gelatinases (MMP-2 and 9), stromelysins (MMP-3, 10, 11), elastases (MMP-7 and 12), and membrane type MMPs (MT-MMPs, MMP-14, 15, 16 and 17) which are surface-anchored (Demedts et al., 2005).

Since MMPs can cause significant damage, they are tightly regulated. Firstly, they are rarely stored but require gene transcription before secretion, the exception being neutrophil MMP-8 and 9. Secondly, they are either secreted as pro-enzymes that require proteolytic cleavage or, in the case of MT-MMPs, activated intracellularly by pro-protein convertases such as furin, that exposes the catalytic cleft, a mechanism known as the cysteine switch. Thirdly, specific inhibitors of MMPs – the tissue inhibitors of metalloproteinases (TIMPs) – are secreted and bind MMPs to prevent enzymatic activity. The balance of MMPs to TIMPs therefore determines matrix turnover, where either an excess of MMPs or a deficit of TIMPs may result in excess ECM degradation (Demedts et al., 2005).

Many inflammatory cells including macrophages, neutrophils, eosinophils, T cells and resident cells such as fibroblasts, airway epithelial cells and airway smooth muscle cells in the airways are capable of synthesising and releasing MMPs. The majority of MMPs are not expressed in normal healthy tissues but are expressed in diseased tissues that are inflamed or undergoing repair and remodelling. MMP expression may be upregulated by exogenous stimuli, cytokines and cell–cell contact. The MMP-2 and MMP-9, referred to as gelatinases, belong to the best-studied MMPs associated with the development of allergic disorders (Vandenbroucke et al., 2011). Increased levels of active MMP-9 and MMP-2 have been detected in sputum samples of asthma patients (Cataldo et al., 2000). Their role pertains to the degradation of type IV collagen, the main component of basal membranes; this facilitates the influx of cells to the site of allergic inflammation (Vandenbroucke et al., 2011).

iii) A disintegrin and metalloproteinases (ADAMs)

The ADAMs are a family of transmembrane and secreted proteins of approximately 750 amino acids in length, with functions in cell adhesion and proteolytic processing of the ectodomains of diverse cell surface receptors and signalling molecules (Edwards et al., 2008). Though all ADAMs contain metalloproteinase domains, in humans only 13 of the 21 genes in the family encode functional proteases (Edwards et al., 2008). Dysregulation of ADAMs expression has been reported in different types of pathologies such as asthma. In the lung, bronchial epithelial cells are capable of producing different types of ADAMs (Dijkstra et al., 2009). ADAM33 was the first member of the ADAM family to be linked to asthma and was the first reported asthma susceptibility gene identified by positional cloning (Van Eerdewegh et al., 2002). Surprisingly little is known about the contributions of ADAM metalloproteinases to pathologies occurring in the airways of asthmatic patients.

iv) Increased vascularity

There is documented evidence for increased vascularity (angiogenesis) and vascular remodelling in asthma (Ribatti et al., 2009) as increased vascularity in the airways has been recognized not only in patients with severe asthma but also in those with stable asthma (Li and Wilson, 1997). Angiogenesis, the formation of new blood vessels from pre-existing ones, has been recognized as an important event in the development and perpetuation of airway inflammation and tissue remodelling in asthma. Vascular endothelial growth factor (VEGF), also known as vascular permeability factor, plays a fundamental role in physiological and pathophysiological forms of angiogenesis (Ribatti et al., 2009). The lung is one of the major organs where VEGF controls several crucial physiological functions. During airway growth, the lung progressively acquires a rich blood supply and this growth is paralleled by the expression of VEGF and its receptors (VEGFRs). VEGF is synthesized by alveolar epithelial cells, bronchial epithelial cells, smooth muscle cells, fibroblasts and alveolar macrophages (Ribatti et al., 2009). Clinical evidences for VEGF involvement in asthma has been shown using induced sputums of asthma patients (Asai et al., 2003) whilst bronchial biopsy specimens from asthma patients has identified increased presence of VEGF+ cells (Chetta et al., 2005) compared to healthy controls.

Chlamydia pneumoniae

History and taxonomy

The order Chlamydiales encompasses a large group of bacteria characterized by their obligate growth in eukaryotic cells (AbdelRahman and Belland, 2005). Chlamydiales currently comprises four families: *Chamydiaceae, Parachlamydiaceae, Simkaniaceae,* and *Waddliaceae* (Pospischil, 2009). *Chlamydiaceae* are the etiological agents of many important human and animal diseases as there are three species of the *Chlamydiaceae* family that infect humans; however, they differ in their tissue tropism, spectrum of disease, genomic content, and morphology. In humans, the genital serovars of *Chlamydia trachomatis* are the most prevalent cause of sexually transmitted disease worldwide while the ocular serovars result in blinding trachoma in developing countries (AbdelRahman and Belland, 2005). *Chlamydia pneumoniae* is a widespread respiratory pathogen and chronic infections are associated with an enhanced risk of developing atherosclerotic, cerebrovascular, and chronic lung disease (AbdelRahman and Belland, 2005).

C. pneumoniae is a ubiquitous, obligate, intracellular, Gram-negative bacterium that commonly causes acute respiratory illness. It has been estimated that most people have two or three *C. pneumoniae* infections during their lifetime (Pasternack et al., 2005) and is transmitted by person-to-person contact through respiratory secretions. *C. pneumoniae* belongs to the *Chlamydiae* group of obligate intracellular pathogens that are characterized by their biphasic developmental cycle (Moulder, 1991). It has a unique developmental cycle (Figure 1.3) with morphologically distinct infectious and reproductive forms: the elementary body (EB) and reticulate body (RB). Upon infection of host cell, the infectious EBs, which are 0.3 -0.35 µm in diameter, attach to the host cell by a process of electrostatic binding and are taken into the cell by induction of active phagocytosis (Wolf et al., 2000). Once internalized, the EBs are contained in membrane-bound cytoplasmic vacuole termed inclusion. At about 8 hours after infection, the EBs begin to undergo profound

changes in morphology to form metabolically active RBs. The resulting RBs multiply by binary fission inside the host cell. At around 36 hours after infection, some RBs begin to reorganize back into EBs through transitional, intermediate forms until 60 hours after infection. Most RBs continue to multiply until the host cell cytoplasm is almost filled by the inclusion, which expands in size concurrently with increase of the chlamydial population. The developmental cycle ceases at 72h at which point the EBs are released by extrusion and/or cytolysis (Hybiske and Stephens, 2007), releasing EBs that infect new host cells (Miyashita et al., 1993; Wolf et al., 2000).

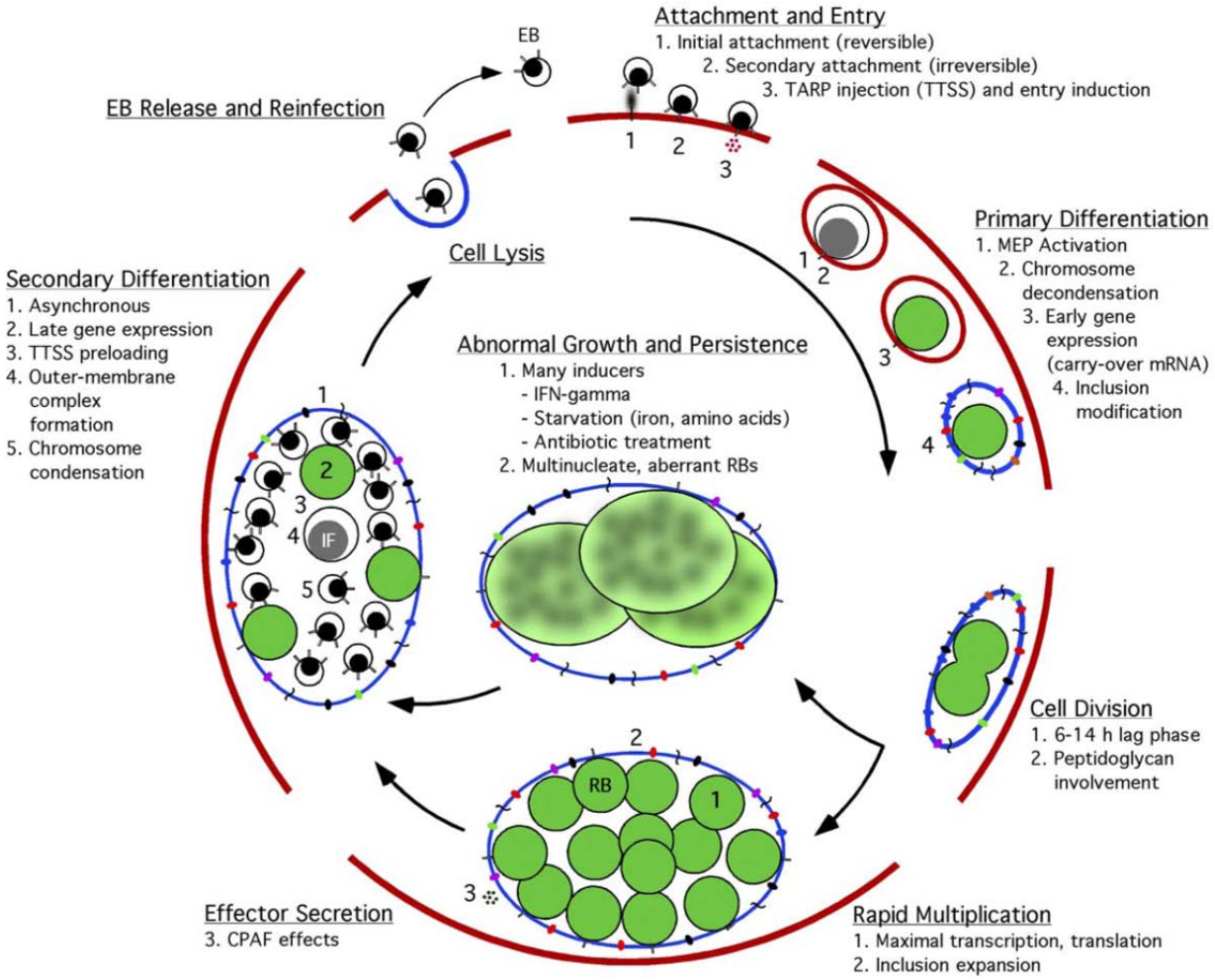

Figure 1.3: *Chlamydia* development cycle inside a human host cell. It depicts the attachment of EBs to the host cell (shown in red line) and the major events that occur inside the host cell as part of the chlamydial development cycle. Figure taken from (AbdelRahman and Belland, 2005).

Although the chlamydial developmental cycle involves progression from EBs to RBs, chlamydial diseases are often associated with a long term or chronic infectious state leading to chlamydial persistence in the host cell. Chlamydial persistence is defined as a long-term association between *Chlamydia* and the infected host cell where *Chlamydia* remains in a viable but noncultivable growth stage (AbdelRahman and Belland, 2005). Persistence occurs due to arrest in chlamydial developmental cycle inside the host cell and it can be induced by a range of factors that stress the chlamydial developmental cycle in some way leading to a shutdown in production of EBs. Such factors include amino acid deprivation in general but tryptophan starvation in particular, iron

depletion, nonlethal antibiotics that target bacterial protein or RNA synthesis, cytokines such as IFN-γ, phage infection, heat shock and growth in monocytes (Hogan et al., 2004). During chlamydial persistence in the host cell, growth characteristics of *Chlamydia* are also altered where chlamydial RBs appears as significantly enlarged and aberrant in shape, instead of the normally round 0.5–1.0 μm RBs, when viewed using an electron microscope (Wolf et al., 2000). However, these changes are reversible and the chlamydial developmental cycle continues normally if inhibitory factors are removed (Hogan et al., 2004).

Chlamydia pneumoniae can infect different human cell types in the human airways such as airway smooth muscle, epithelial cells, macrophages and endothelial cells (Blasi et al., 2009) leading to unlimited proliferation without killing infected host cells (Kim et al., 2009). After being released into the extracellular environment, the elementary bodies interact with respiratory epithelial cells, leading to inclusion formation and subsequent intracellular replication (Metz and Kraft, 2010). The complete developmental cycle takes between 36 and 72 h, depending on the chlamydial species but in *C. pneumoniae* this is typically 72 hr (Campbell and Kuo, 2009).

Chlamydia pneumoniae and asthma

With regard to its unique spectrum of disease associations, *C. pneumoniae* differs considerably from other species of *Chlamydia* as it is associated with variety of chronic diseases such as Reiter's syndrome (Seals and Courtneidge, 2003), asthma (Hahn et al., 1991), chronic obstructive pulmonary disease (COPD) (Fort et al., 2001), multiple sclerosis (Moro et al., 2010), Alzheimer disease (Fallon et al., 2006) and atherosclerosis (Li and Hendriks, 2013). Accumulating evidence from sero-epidemiological studies has shown that many asthmatics have elevated antibody levels to *C. pneumoniae*, suggestive of persistent infection (Biscione et al., 2004; Black et al., 2000; Johnston and Martin, 2005; Pasternack et al., 2005; von Hertzen, 2002).

Hahn et al. (Hahn et al., 1991) was the first to show the association between *C. pneumoniae* and adult-onset asthma. In the study by Hahn and colleagues (Hahn et al., 1991) 365 patients with diagnosis of pneumonia were enrolled. Patients had their serum samples collected, clinical data recorded and throat culture specimens were collected from 90.4% of the patients. Patients were also asked to return in four weeks time for another serologic testing. Serum samples from the patients were used for diagnosis of negative, preexisting, acute and chronic phase of *C. pneumoniae* infection in the enrolled patients. Results revealed that 9 out of 19 enrolled patients with acute *C. pneumoniae* infection had bronchospasm and strong correlation was shown between patients with wheeze and increasing *C. pneumoniae* antibody titers. A matched comparison study was also performed on those enrolled patients where patients were divided into two groups: i) exposed patients who had *C. pneumoniae* antibody titer of at least greater than 1:64 and ii) unexposed patients who had *C. pneumoniae* titer of less than 1:16. This was done to test the hypothesis of a significant association between *C. pneumoniae* exposure and the clinical diagnosis of asthmatic bronchitis. Results revealed that twenty-one (29.6%) of 71 exposed patients and five (7%) of 71 unexposed matched control subjects had a diagnosis of asthmatic bronchitis after respiratory illness. This proved a strong, statistically significant association of *C. pneumoniae* exposure and the diagnosis of asthmatic bronchitis.

Hahn and colleagues have further examined the association of *C. pneumoniae* infection and asthma in another study (Hahn and McDonald, 1998), where they sought to determine whether patients with acute *C. pneumoniae* respiratory tract infections would develop chronic asthma. The study (Hahn and McDonald, 1998) enrolled 163 adult patients who are diagnosed with chronic asthma and/or recorded to have a first-ever wheezing episode. Only 10 of the 163 enrolled patients were found to have a first ever wheezing episode. The patients were diagnosed with *C. pneumoniae* infection by means of both microbiological and serological testing. Results revealed that *C. pneumoniae* infection was diagnosed in 20 (12%) of the 163 enrolled patients. Moreover, half (5 of 10) of the patients recorded with first ever wheezing episode had acute *C. pneumoniae* infection (diagnosed by standard serologic criteria) and subsequently developed chronic asthma. This supported the observation that acute *C. pneumoniae* infection can lead to the development of chronic asthma (Hahn and McDonald, 1998).

Since the study by Hahn and colleagues first proved the association between *C. pneumoniae* infection and asthma development, there have been numerous studies (Agarwal and Chander, 2008; Awasthi et al., 2012; Biscione et al., 2004; Black et al., 2000; Cosentini et al., 2008; Hahn et al., 2012; Johnston and Martin, 2005; Patel et al., 2012; von Hertzen, 2002) supporting the association between *C. pneumoniae* and asthma. However, there have also been few studies (Dejsomritrutai et al., 2009; Larsen et al., 1998; Pasternack et al., 2005) disproving the association between *C. pneumoniae* infection and asthma development. One such study has been undertaken by Pasternack and colleagues (Pasternack et al., 2005) who followed a population-based adult cohort for 15 years to find out whether there is an increased asthma risk in subjects with previous or chronic *C. pneumoniae* infection. The study by Pasternack and colleagues (Pasternack et al., 2005) made a clinical evaluation of patients with new persistent asthma (n=83) and matched controls (n=162), who were selected from a population-based adult cohort. Spirometry testing and skin prick testing was done on the subjects to evaluate for pulmonary function and asthma diagnosis. Meanwhile, serological testing was also performed on the patients for diagnosis of *C. pneumoniae* infection. Results revealed that elevated *C. pneumoniae*–specific antibody titers did not increase the probability of new persistent asthma. However subjects, with chronic *C. pneumoniae* infection and newly diagnosed asthma, experienced a faster decline in FEV_1 compared with other asthmatics without chronic infection. This suggests that *C. pneumoniae* infection could contribute to asthma development by promoting airway remodelling in infected patients.

More recently, new studies have been published on the presence of *Chlamydia* specific-IgE in asthma patients (Hahn et al., 2012; Patel et al., 2012). Elevated IgE levels are associated with asthma as IgE binds to FcεR1 receptors on basophils and mast cells triggering release of inflammatory mediators (Wu, 2011). Infection with *C. pneumoniae* induces serum immunoglobulin M (IgM), IgA, and IgG responses (Kuo et al., 1995). A study by Patel and colleagues (Patel et al., 2012) have identified the presence of *C. pneumoniae*- specific IgE antibodies in a cohort of children with chronic respiratory disease. As per the study (Patel et al., 2012), serum and broncho alveolar lavage (BAL) samples were isolated from 197 patients aged between 0-20yo. BAL samples were used for detection of chlamydial DNA by PCR and were also used for analysing chlamydial viability by tissue culture techniques. Meanwhile, serum samples were analysed by PCR for detection of chlamydial DNA. Total IgE levels were evaluated from both the serum and BAL samples while western blot was used to determine whether these IgE was *C. pneumoniae* specific. Results from the patient cohort revealed that 38% (74/197) were culture positive for *Chlamydia* with majority of the *Chlamydia*

culture-positive patients, 84% (62/74), were also diagnosed with chronic, severe asthma according to GINA (Global Initiative for Asthma) guidelines. In terms of *Chlamydia* specific serum IgE antibody levels in the patient cohort, 54% (107/197) had *Chlamydia*-specific IgE whilst suffering from chronic respiratory disease while the healthy controls did not reveal any presence of Chlamydia-specific IgE. Collectively, these results suggest that through production of *Chlamydia*-specific IgE, *C. pneumoniae* can play a direct role in driving asthma pathogenesis (Patel et al., 2012).

Parallel findings from Patel at al. (Patel et al., 2012) were also reported in another study by Hahn and colleagues (Hahn et al., 2012) which measured the prevalence of *C. pneumoniae*-specific IgE in a sample of community adult asthma patients. This was done using case-control studies of the association of *C. pneumoniae* IgE and asthma using healthy blood donors and non-asthmatic clinic patients as controls. As per the study (Hahn et al., 2012), 66 patients with physician-diagnosed asthma were enrolled and blood samples were isolated for detection of *Chlamydia* by PCR and assessment of *Chlamydia* specific-IgE levels from serum samples. *Chlamydia*-specific IgE from the serum samples were identified by means of western blot assay. Results revealed that 50% (33/66) of the patient cohort had the presence of *C. pneumoniae* IgE while 24% (16/66) had the presence of *Chlamydia* DNA. Since total serum IgE has been associated with asthma (Galli and Tsai, 2012), this study concluded that a significant (P=0.001) association was found between *C. pneumoniae* IgE and asthma diagnosis. Collectively, these studies (Hahn et al., 2012; Patel et al., 2012) demonstrate that *C. pneumoniae* is frequently present in the respiratory tract of paediatric asthma patients and may be involved in the development of asthma in chidren.

To sum up, clinical evidence for the association between *Chlamydia pneumoniae* and asthma in humans has been largely based on sero-epidemiological studies (Awasthi et al., 2012; Biscione et al., 2004; Black et al., 2000; Gencay et al., 2001; Hahn et al., 1991; Hahn and McDonald, 1998; Hahn et al., 2012; Johnston and Martin, 2005; Metz and Kraft, 2010; Patel et al., 2012; von Hertzen, 2002) where an increased prevalence of antibody titers to *C. pneumoniae* were detectable in asthma patients. Meanwhile, recent studies by Patel et al (Patel et al., 2012) and Hahn et al (Hahn et al., 2012) have shown evidence for seroprevalence of *C. pneumoniae*- specific IgE antibodies are associated with asthma. However, none of these studies has been able to fully deduce the causal mechanism behind *C. pneumoniae* infection and asthma. Furthermore, none of the studies have assessed specific cytokine responses of immune cells to in *vitro exposure* to *Chlamydia*. Hence, more study is needed to evaluate whether *C. pneumoniae* infection is a pathogenic factor in asthma or merely an epiphenomenon that is somehow related to the enhanced airway inflammation seen in subjects with chronic asthma.

Mechanism of asthma predisposition by *C. pneumoniae* in mice

Currently, murine models of chlamydial lung infection have been valuable in understanding the mechanism by which *C. pneumoniae* can drive the asthmatic airway response since murine models of chlamydial lung infection are highly representative of the human condition (Hansbro et al., 2004). Asthma responses in mice can be experimentally induced through exposure to ovalbumin (OVA) antigen (Asquith et al., 2008; Horvat et al., 2007; Horvat et al., 2010a; Horvat et al., 2010b; Kaiko et al., 2008; Pouliot et al., 2010) while the mouse pneumonitis biovar of *C. muridarum* is usually the

choice of pathogen for studying *C. pneumoniae* infection in murine asthma models (Hansbro et al., 2004).

So far, studies from murine models have shown that chlamydial infection of DCs *in vitro* can demonstrate distinct inflammatory responses which are skewed towards a Th2 phenotype (Kaiko et al., 2008). Furthermore, the injection of *Chlamydia*-infected DCs into the airways of naive mice induced all features of asthma which demonstrates the *in vivo* capacity of *Chlamydia*-infected DCs to initiate allergic airway inflammation. Hence, this unique and potent Th2 adjuvant characteristic of *Chlamydia* which are partially mediated through DCs, may explain the mechanism behind this strong association between *Chlamydia* and asthma.

Murine studies have also shown that early life chlamydial infection can predispose to asthma in later life during adulthood (Horvat et al., 2007; Horvat et al., 2010b). Studies by Horvat et al (Horvat et al., 2007; Horvat et al., 2010b) demonstrated that chlamydial infection during the neonatal and infant stage of mice was able to induce the hallmark features of asthma such as induction of AHR, secretion of Th2 cytokines, enhanced mucus secretion, eosinophil infiltration in the lung and increased number of activated DCs. However, adult mice infected with *Chlamydia* did not demonstrate the hallmark features of asthma. Moreover, analysis of lung structures revealed that a decrease in lung function in neonates with deleterious alterations in airway resistance and compliance (Kaiko et al., 2008). A plausible explanation could be that the lungs, unlike most other organs, continue to mature during the first two years of life. Thus, early-life lung infections may permanently affect pulmonary immunity, function and structure which might predispose to asthma in later life. Findings from Horvat et al (Horvat et al., 2007; Horvat et al., 2010b) was also replicated in another independent study by Jupelli et al (Jupelli et al., 2011) who showed that respiratory chlamydial infection (100 IFUs) in neonatal mice leads to respiratory dysfunction in adult life. However, administration of antimicrobial treatment erythromycin during the neonatal chlamydial infection induced early bacterial clearance and partially ameliorated the *Chlamydia*-induced lung dysfunction as adults. This suggests the importance of antimicrobial administration in neonatal chlamydial respiratory infection in preventing the progression of respiratory dysfunction in adulthood.

Hematopoietic cells has been suggested to play a role behind this asthma predisposition in infant mice (Starkey et al., 2012a). A study by Starkey and colleagues (Starkey et al., 2012a) have shown that upon transfer of infected bone marrow cells into recipient age matched irradiated naive mice resulted in induction of allergic airway disease along with increased Th2 cell cytokine release (IL-5 and IL-13) and IL-13 levels in the lung. This effect was evident in infant (3-weeks-old) mice but not neonatal (24-hours-old) and adult (6-weeks-old) mice. Hence, this suggests that an infant chlamydial lung infection results in long lasting alterations in hematopoietic cells that increases the severity of allergic airway disease in later-life (Horvat et al., 2007; Starkey et al., 2012a).

Two hypotheses have been proposed to explain this association between *C. pneumoniae* infections and asthma (Horvat et al., 2007). One hypothesis is that early-life infection with *C. pneumoniae* may permanently affect the pulmonary immune system as well as its function since early-life responses to infection are highly polarized toward Th2 immunity (Horvat et al., 2007). This can partly be attributed to the inability of DCs to produce IL-12, a key Th1-trophic cytokine, during early childhood (Langrish et al., 2002; Upham et al., 2002). It has been shown that human DCs obtained from neonates, 5-year old and 12-year old children have impaired ability to synthesise IL-12 in response to

microbial stimuli. In contrast, human adult DCs can produce significant amount of IL-12 in response to microbial stimulation which suggests that the maturity of DCs could be the key rate-limiting step in the postnatal development of Th1 function (Upham et al., 2002). It is, therefore, possible that a chlamydial lung infection in early life can reinforce aberrant Th2 responses to infection with *C. pneumoniae*. This may cause the immune system to mature with a more allergic phenotype that fails to clear the bacteria, resulting in persistent infection and increasing the severity of Th2-type inflammatory responses to environmental antigens which in turn may promote asthma in susceptible individuals. Another explanation is that Th1-inducing infections, such as *C. pneumoniae*, may cause a generalized inflammation of the airways that leads to the exacerbation of allergen-induced inflammation and asthma (Horvat et al., 2007).

Recently, there has been some advancement in studying the association of chlamydial respiratory tract infections in triggering asthma in murine models. A study by Crother and colleagues (Crother et al., 2011) has shown that *Chlamydia pneumoniae* infection can induce development of allergic airway sensitization in mice and deduced the mechanism for this allergic airway inflammation. As per the study (Crother et al., 2011), mice were exposed to Human serum albumin (HSA) in combination with *C. pneumoniae* or PBS control intranasally and then re-exposed to HSA two weeks later. Results revealed that upon re-exposure to HSA, mice that were initially exposed to HSA and *C. pneumoniae* developed significant inflammation in the lungs as determined by H&E (hematoxylin and eosin) staining. In contrast, mice that received HSA without *C. pneumoniae* during sensitization did not develop pulmonary inflammation.

To deduce whether this pulmonary inflammation seen in the mice was due to TLR2 and TLR4 signalling, the authors (Crother et al., 2011) utilised TLR2 and TLR4 knockout mice which were infected with *C.pneumoniae*, sensitized with HSA for three days (beginning five days after infection), and challenged with HSA later. On observation, it was shown that the TLR2 knockout mice developed higher pulmonary pathology compared to the wild type while the TLR4 knockout mice developed lower pulmonary pathology compared to the wild type. This suggests that *C. pneumoniae*-induced allergic sensitization requires TLR4 but not TLR2 signalling. To understand this differential allergic sensitization observed between TLR2 and TLR4 knockout mice, the authors sought to look at the number of regulatory T-cells (Tregs) in the lungs of mice. It revealed that baseline levels of Tregs in the lung were significantly reduced in TLR2 knockout mice compared to wild type and TLR4 knockout mice during chlamydial infection which suggested a protective effect of Tregs against chlamydial infection.

Having deduced the mechanism of *C. pneumoniae*-induced pulmonary inflammation in mice, the authors (Crother et al., 2011) decided to look at the effect of *C. pneumoniae* dosage in the development of allergic inflammation. Mice were infected with both low-dose (5×10^5 IFU) and high-dose (5×10^6 IFU) of *C. pneumoniae* and five days later immune cells present in the lungs were analysed by flow cytometry. On analyses, it was observed that a dramatic and significant increase of Tregs and pDCs (plasmacytoid dendritic cells) was present in the lungs of mice in the high-dose infection group, compared to low-dose infection group and uninfected control group. This suggests that both Tregs and pDCs play a suppressive role towards antigen sensitization. Hence, the results from this study suggests that chlamydial respiratory tract infection can provide an adjuvant effect in asthma development through TLR4 signaling pathway while same infection can result in reduced or

greater numbers of Tregs, which may ultimately control whether allergic sensitization occurs or not. Apart from preventing antigen sensitization, pDCs have also been shown to play a role in immune responses against *C. pneumoniae* infection in mice (Crother et al., 2012). Their study (Crother et al., 2012) have shown that depletion of pDCs during a *C. pneumoniae* infection in mice resulted in delayed lung inflammation and bacterial clearance while increasing the pDC numbers in the lung by Flt3l (Fms-related tyrosine kinase 3 ligand) treatment experimentally results in greater lung inflammation during acute *C. pneumoniae* infection (Crother et al., 2012). Meanwhile, restimulation of T-cells in the draining lymph nodes of pDC-depleted mice induced greater amounts of proinflammatory cytokines than observed in control mice. These results suggest that pDCs in the lung may provide critical proinflammatory innate immune responses in response to *C. pneumoniae* infection, but are suppressive towards adaptive immune responses in the lymph node (Crother et al., 2012).

The immune system needs to effectively activate innate immune cells to clear a pathogen, whilst it also needs to maintain an effective system of self-regulation to inhibit an unbalanced immune response and collateral damage of host tissue (Akira, 2011). Studies examining mouse models reveal that inefficient immune system responses can escalate respiratory disease pathology following chlamydial respiratory infection which can also lead to less optimal clearance of *Chlamydia*. IL-13 is a key cytokine in asthma as it is known to have pleiotropic effects in driving asthma pathology. Keeping this in mind, a study by Asquith and colleagues (Asquith et al., 2011) decided to look at the role of IL-13 in chlamydial lung infection in mice. Their studies (Asquith et al., 2011) have shown that release of IL-13 in lungs of mice following chlamydial infection results in enhanced allergic inflammation. As per the study, adult wild type mice and IL-13 knockout mice were infected intranasally (100 IFU) with *Chlamydia muridarum* (mouse pneumonitis biovar) for 20 days and *C. muridarum* load in the lungs was monitored during this 20 day period. Results revealed that the *C. muridarum* load in the lungs was significantly lower in the IL-13 knockout mice when compared to the wild type infected group. Moreover, on analysing the pulmonary inflammation in the lungs it showed that the IL-13 knockout mice had a significant reduction in total leukocyte, neutrophil, macrophage and lymphocyte influx into the lungs compared to wild type controls. To deduce whether IL-13 was produced by CD4 T cells in mice, the authors experimented further where they injected mice with CD4 T cell depleting antibody prior to and after intranasal infection with *C. muridarum*. Results showed that chlamydial load in the lungs was still higher in the wild type infected mice when compared to the IL-13 knockout mice suggesting that the source of IL-13 in response to chlamydial infection is linked to the innate host defence response. To investigate the mechanism behind this efficient chlamydial clearance seen in IL-13 knockout mice, the authors of the study (Asquith et al., 2011) isolated alveolar macrophages from the Broncho alveolar lavage fluid (BALF) of infected wild type and IL-13 knockout mice and assessed the engulfment of *C. muridarum* by staining them using a *Chlamydia* specific fluorescent labelling kit. Results revealed that only half as many BALF macrophages from wild type mice stained positive for *C. muridarum* compared to those from IL-13 knockout mice which suggests that that in the absence of IL-13, the uptake of *C. muridarum* by macrophages is enhanced. Thereby, the authors of the study (Asquith et al., 2011) concluded that IL-13 responses to chlamydial respiratory infection are important in promoting inflammation in the respiratory tract where presence of IL-13 contributes to reduce macrophage phagocytosis and enhanced infection of airway epithelial cells.

The ability of the innate immune system to quickly recognize and respond to an invading pathogen is essential for controlling the infection. For this purpose, cells of the immune system express receptors which recognize evolutionarily conserved structures expressed by various pathogens but absent from host cells (Akira, 2011). That said, it is possible that chlamydial respiratory infection can modulate immune responses through interaction with Toll-like receptors (TLRs) on innate immune cells. Interaction of *C. pneumoniae* with TLRs promotes expression and secretion of inflammatory cytokines, which can mount the earlier innate immune response and subsequent adaptive immune responses (Prebeck et al., 2001). Recognition of *C. pneumoniae* by TLRs is mediated primarily by TLR2 and TLR4 where recognition of endotoxin appears to be mediated predominantly through TLR4, whereas TLR2 appears to be involved in the recognition of lipoproteins (Prebeck et al., 2001). Both TLR2 and TLR4 are known to be expressed intracellularly in human dendritic cells (Uronen-Hansson et al., 2004) which enables dendritic cells to detect intracellular *Chlamydia*. Recent studies from murine models has shown that activation of TLR2, but not TLR4, is required for inducing an efficient immune response against chlamydial respiratory infection in neonatal mice (Beckett et al., 2012) while absence of TLR2 activation can lead to enhanced pathology in the lungs in response to infection (He et al., 2011). The study by Beckett et al. (Beckett et al., 2012) utilised neonatal wild-type, TLR2 deficient (TLR2-/-), TLR4 deficient (TLR4-/-) and TLR2/4 double knockout (TLR2/4-/-) mice which were intranasally challenged with *C. muridarum* (400 IFU). Upon observation it revealed that the TLR2-/- mice had increased influx of inflammatory cells in the lungs combined with reduced phagocytic activity of neutrophils which led to increased pathology in the lungs. These results were also observed in another independent study by He at al. (He et al., 2011) who showed that intranasal challenge with *C. muridarum* (5000 IFU) in TLR2-/- mice led to increased pathology in lungs due to an increase in production of inflammatory cytokines, such as TNF-α, IFN-γ, IL-17 and RANTES, along with impaired bacterial clearance in the lungs. It could be argued that the increased levels of cytokine IFN-γ seen in TLR2-/- mice (He et al., 2011) could possibly be due to impaired expansion of CD4+CD25+Foxp3+ regulatory T-cells (Tregs) (Wantia et al., 2011). A study by Wantia et al. (Wantia et al., 2011) has shown that intranasal challenge (2.5 X 10^6 IFU) with *C. pneumoniae* in TLR2/4 double knockout (TLR2/4-/-) led to enhanced secretion of IFN-γ by pulmonary CD4 T cells combined with an increase in *Chlamydia pneumoniae*-induced loss of body weight as opposed to the wild-type. Upon analyses of pulmonary immune cells from TLR2/4-/- mice, the presence of lower frequency of Tregs was noted. Hence, it shows that impaired expansion of Tregs accompanied by increased frequency of CD4+IFN-γ+ effector T-cells contributes to the pulmonary damage in TLR deficient mice (Wantia et al., 2011).

The airway epithelium is responsible for forming a barrier between the environment and the underlying host tissues and is often proposed to take an active role in the innate immune response to pathogens. Studies examining chlamydial growth in murine pulmonary epithelial cell line has shown that IL-17 acts in synergy with IFN-γ to induce production of inducible nitric oxide synthase (iNOS) which leads to inhibition of chlamydial growth in murine epithelial cell line (Zhang et al., 2012). The study by Zhang et al (Zhang et al., 2012) has shown that addition of IL-17 and IFN-γ to chlamydia muridarum infected murine lung epithelial cell line TC-1 leads to significant production of iNOS and nitric oxide (NO) which acts to inhibit chlamydial growth in murine epithelial cell line and this same effect was also observed in murine macrophage cell line RAW 264.7 primary peritoneal macrophages. Moreover, upon neutralization of IL-17 in following *C. muridarum* lung infection (1000 IFUs) led to significant reduction in iNOS expression along with higher bacterial loads in the lung.

This suggests that IL-17A and IFN-γ play a synergistic role in inhibiting chlamydial lung infection, at least partially through enhancing iNOS expression and NO production in pulmonary epithelial cells and macrophages.

In summary, numerous animal studies have outlined mechanisms by which chlamydial respiratory infections may promote allergic lung inflammation. The study by Crother and colleagues (Crother et al., 2011) has shown how infection with *C. pneumoniae* can promote asthma development through TLR4 signaling pathway but on the other hand, the study by others (Beckett et al., 2012; He et al., 2011) have shown TLR2 signaling pathway is required for effective immunity against chlamydial respiratory infection. Moreover, these studies also showed how the presence of Tregs can prevent asthma development (Crother et al., 2011) while a decrease in Treg cell population in lungs can contribute to increased pathology in the murine lungs (Wantia et al., 2011). Both Th1 and Th17 responses are required for effective clearance of chlamydial respiratory infections (Gao et al., 2012) with Th1 and Th17 cell derived cytokines act on airway epithelial cells and macrophages (Zhang et al., 2012) to inhibit chlamydial lung infection. As can be surmised in understanding these effects of chlamydial respiratory infection on the immune response in murine models, it seems that a combination of presence of Treg cells and pDCs and an imbalanced TLR2 and TLR4 signaling pathway can affect development of asthma in response to *C. pneumoniae* infection. However, in humans such a conclusion is yet to be derived as studies are required to fully deduce the link between *C. pneumoniae* and asthma.

Dendritic cells (DCs)

Dendritic cells (DC) are highly efficient antigen-presenting cells (APC) that are specialized for the capture, processing and presentation of antigens to T cells (Shortman and Naik, 2007). The function of DCs falls into two categories, all of which share the common feature of antigen presentation but differ in performing distinct immune function (Naik et al., 2007). The first category of DC function is antigen presentation and activation of T cells while the second category of DC function is the induction and maintenance of immune tolerance.

DC origin and subtypes

DCs, like other immune cells, are derived from hematopoietic stem cells through early progenitor cells which leads to development of different subsets of DCs (Caux and Dubois, 2001). These subsets of DCs have unique phenotypes and functional potentials (Dakic and Wu, 2003). The lifecycle of DCs start with their development from CD34+ hematopoietic progenitors in the bone marrow, progressing from a macrophage and DC precursor (Figure 1.4) capable of giving rise to both lineages to a common DC progenitor generating DC subsets but not monocytes/macrophages (Soloff and Barratt-Boyes, 2010). These subsets of DCs have locations throughout the immune network but they do not necessarily share a common DC phenotype or functional role. The complexity of DC subsets reflects their plasticity and ability to perform different functions in different environments, thereby enabling their bipolar effects on the immune response.

In peripheral blood, two subsets of DC populations have been identified namely, myeloid DC (mDC) and plasmacytoid DC (pDC), which differ in their developmental pathways. DC development, particularly the commitment to a specific DC subset, is regulated by a number of key cytokines such

as fms-like tyrosine kinase 3 (Flt3) ligand (Naik et al., 2007). In the total absence of GM-CSF, Flt3 preferentially promotes pDC development while the presence of both GM-CSF and Flt3 promotes mDC differentiation via STAT-5 dependent mechanisms. Both subsets of DC population can be found circulating throughout the body as well as residing in specific tissues in the body.

Myeloid DCs (mDC) express the receptor for granulocyte macrophage-colony stimulating factor (GM-CSF) and other myeloid cell markers such as CD11c, CD13, and CD33 while pDC, also present in bone marrow and in secondary lymphoid tissues, express the receptor for interleukin 3 (IL-3; CD123) and lack myeloid cell markers. In vitro, peripheral blood DC populations were first described to exhibit different functions: mDCs, also called Type 1 DCs (DC1), produce high levels of IL-12 when stimulated with tumor necrosis factor-α or and drive a potent Th1-polarized immune response. On the other hand, pDCs are important mediators of antiviral immunity through their ability to produce large amounts of type I interferons (IFNs) on viral infection. (Matsuda et al., 2002). Plasmacytoid DCs produce large amounts of interferon (IFN)–α and (IFN)–β in response to viruses which are sensed through expression of TLR7 and TLR9 (Steinbrink et al., 2009). Meanwhile mDCs express Toll like receptor (TLR) 1, 2, 4, 5 and 8 and respond to the appropriate microbial ligands, including peptidoglycan, lipoteichoic acid, flagellin and LPS which corresponds to bacterial stimulation (Dakic and Wu, 2003). These two DC subtypes are short-lived and have to be continuously regenerated from hematopoietic stem cells in the bone marrow. This specialization and division of labor presumably allows the immune system to intricately 'tailor' an adaptive immune response appropriate for the breaching pathogen. Blood monocytes, or CD14 monocytes, are the most commonly used precursor cells for generating human DCs in culture. In the presence of macrophage colony-stimulating factor (M-CSF), they will generate macrophages but, in the presence of GM-CSF and IL-4, DCs are produced after six days (Shortman and Liu, 2002).

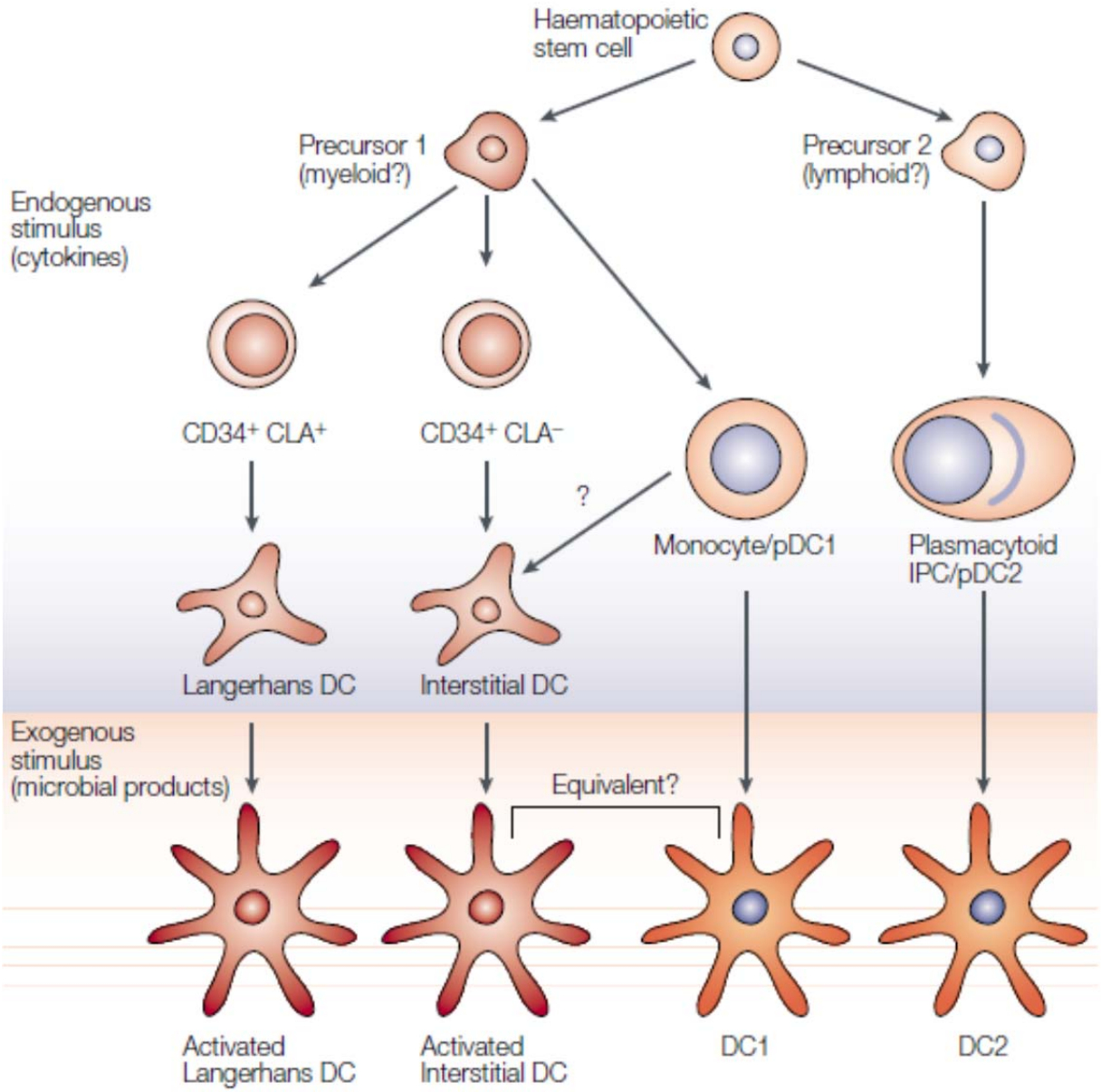

Figure 1.4: Pathways of human DC development. The first step towards the development of the individual types of blood cell is considered to be the formation of precursor cells that have a strong bias towards either the myeloid or the lymphoid lineages. The myeloid precursors and the lymphoid precursors express the FLT3 (FMS-related tyrosine kinase 3) receptor and have the greatest capacity to form DCs (Shortman and Naik, 2007). Some DC lineages, such as Langerhans and interstitial DCs, progress to a mature but quiescent state under the influence of cytokines alone, requiring exogenous stimuli only for full activation. Other DC lineages, such as DC1 and DC2 however, probably remain at a precursor state in an uninfected individual, because they require stimulation by microbial products to produce mature DCs. CLA, cutaneous lymphocyte-associated antigen; IPC, interferon-producing cell; pDC, precursor of DC. Figure taken from (Shortman and Liu, 2002).

DCs and asthma

Following allergen challenge, accumulation of both mDC and pDC has been detected in the sputum (Dua et al., 2010) and airway lumen (Bratke et al., 2007) of asthmatic patients which suggest that both mDCs and pDCs are recruited from peripheral blood to the human respiratory tract after allergen challenge.

DCs possess the ability to uptake antigen, present to and activate, naive T cells thereby initiating adaptive immune responses in the respiratory mucosa. DCs form an elaborate network throughout the lung and can be found throughout the conducting airways, lung interstitium, lung vasculature, pleura, and bronchial lymph nodes (Lambrecht and Hammad, 2009). DCs form tight junctions with airway epithelial cells by expressing occludin and claudin family members as well as zona occludens-1 (ZO-1) protein. In addition, DCs attach to airway epithelial cells via E-cadherin and CD103 expressed by a subset of DCs that probes the airway lumen (Lambrecht and Hammad, 2010). DCs are migratory immune cells as they traffic from one site to another and primarily mediate the antigen uptake and presentation to naive T-cells. DCs are minimally active in absence of allergens as allergens are usually recognized by Toll-like receptors (TLRs) in DCs. Binding of allergens to DCs activate TLRs which enhances motility of DCs and antigen sampling behavior.

DCs display a different functional repertoire at different stages of their development. In an immature state DCs are very effective in processing native protein antigens for the MHC class II restricted pathway. Meanwhile, mature DCs are less able to capture new proteins for presentation but are much better at stimulating naive CD4+ and CD8+ T cells to grow and differentiate. Maturation occurs *in vivo* when immature DCs migrate from areas of antigen uptake to the T cell areas of lymphoid organs (Romani et al., 1996). As pulmonary DCs occupy such a key position in the immune response, there has been much interest in the mechanism by which they interact with aeroallergens and respiratory pathogens.

DCs have been identified in the bronchial biopsy specimens of patients with asthma (Jahnsen et al., 2001). In the study by Jahnsen and colleagues (Jahnsen et al., 2001), a pilot study was conducted in order to examine the potential of DCs to migrate to the airways of asthmatic patients in response to bronchial challenge with specific allergen. The study by Jahnsen and colleagues characterized seven asthma patients who were challenged with increasing concentration of house dust mite solution, through inhalation, until a 20% reduction in FEV_1 from the post-saline value was achieved. Bronchoscopy was also performed on the patients, 1 week before and 4-5 hours after the allergen challenge, to obtain bronchial biopsy samples and analyse them using multicolour immunofluorescence staining. Results revealed that a nearly two-fold increase in number of DCs was observed in the bronchial biopsies of patients following allergen challenge with DCs being identified using the multicolour immunofluorescence staining. These results provided the first evidence for the ability of DCs to accumulate in the human bronchial mucosa in response to allergic inflammation.

Human DCs and *Chlamydia pneumoniae*

Studies have shown that DCs are susceptible to *C. pneumoniae* infection *in vitro* and infection induces secretion of TNF-α which acts to restrict chlamydial growth in DCs (Njau et al., 2009a; Njau et al., 2009b; Wittkop et al., 2006). Secretion of TNFα by *C. pneumoniae*-infected DCs promotes the induction of indoleamine 2,3-dioxygenase (IDO) gene in DCs (Njau et al., 2009a; Njau et al., 2009b) which eventually restricts *C. pneumoniae* replication in DCs. Meanwhile inhibition of TNF-α, by using anti-TNF-α antibody adalimumab, can enhance the multiplication of *C. pneumoniae* in DCs which suggests that IDO induction in DCs is dependent on TNF-α (Njau et al., 2009b). Hence, current data (Njau et al., 2009a; Njau et al., 2009b; Wittkop et al., 2006; Wittkop et al., 2008) suggests that induction of IDO gene expression by TNF-α represents a potential mechanism for DCs to restrict bacterial growth in *C. pneumoniae* infections.

In terms of *C. pneumoniae* growth in DCs, infectious particles and chlamydial transcripts can be detected in human DCs up to 25 days post infection (Kis et al., 2008; Wittkop et al., 2006). This observation was made by identifying transcripts of chlamydial genes encoding products involved in cell division and energy production (dnaA, ftsK, and tal) which were detected by RT-PCR and indicates that a productive infection of *C. pneumoniae* was established (Kis et al., 2008; Wittkop et al., 2006). Further evidence of productive infection by *C. pneumoniae* in DCs was evident through detection of 16S rRNA and groEL-1, a molecular chaperone protein (Wuppermann et al., 2008), were detected in DCs up to 5 days post infection (Wittkop et al., 2008). However, titration of DC lysates on HEp-2 cells showed that infectious progeny recovered at various intervals showed no exponential growth (Kis et al., 2008; Wittkop et al., 2006). Hence, chlamydial development in DCs is not exponential as it is observed in epithelial cells such as HEp-2 and BEAS-2B cells with chlamydial inclusions in DCs differing morphologically from those observed in infected epithelial cells.

Immune responses to *C. pneumoniae* by DCs are driven towards Th1 and Th17 polarisation which were evident through increased production of cytokines IL-1β, IL-6, IL-10 and IL-12p70 (Flego et al., 2012). Infected DCs also switch to a mature phenotype as they display increased expression of CD80 and CD83 along with increased capacity to drive naïve T cell polarisation to Th1 and Th17 cells. Intracellular pathways triggered by *C. pneumoniae* involved TLR2 signaling as addition of TLR2 inhibitors reduced IL-10 and IL-12p70 production by DCs along with a significant expansion of Th2 cells whilst inhibiting Th1 and Th17 cells. Hence, *C. pneumoniae* infection of DCs triggers TLR2 signalling pathways which consequently leads to production of a set of pro-inflammatory cytokines along with polarization towards Th1 and Th17 responses (Flego et al., 2012).

In conclusion, data from current literature suggests that human DCs are susceptible to *C. pneumoniae* infection (Wittkop et al., 2006) but DCs respond through an increased production of TNF-α (Ausiello et al., 2006; Kis et al., 2008; Njau et al., 2009b; Wittkop et al., 2006; Wittkop et al., 2008)and IDO (Njau et al., 2009a) which act to restrict bacterial metabolism leading to chlamydial growth arrest and persistence. Moreover, DCs responses to *C. pneumoniae* are directed towards Th1 and Th17 response which are mediated by TLR2 signaling pathways (Flego et al., 2012).

CD4+ T helper type 2 (Th2) cells, Type 2 innate lymphoid cells (ILC2) and asthma

A currently widely accepted model of asthma is of a disease caused by chronic inflammation of the airways, directed largely by Th2 lymphocytes reacting to inhaled allergens and antigens (Wardlaw et al., 2002). Th2 cells perform pleiotropic activities in asthma through the release of various cytokines particularly interleukin 4 (IL-4), IL-5, IL-9 and IL-13 and Th2 cells have been identified in the bronchoalveolar lavages (BAL) and airways biopsies of asthma patients (Robinson et al., 1993a; Robinson et al., 1993b). In contrast, Th1 cells preferentially produce IL-2 and interferon gamma (IFN-γ), which promotes cellular immune responses including the activation of cytolytic T cells, and the killing of intracellular pathogens by macrophages (Renauld, 2001). IFN-γ production from Th1 cells inhibits proliferation of Th2 clones while IL-10 production from Th2 cells inhibits proliferation of Th1 clones (Robinson et al., 1992). Th1 and Th2 lymphocytes differ on the basis of their cytokine

secretion profile and functional capabilities (Constant and Bottomly, 1997; Murphy and Reiner, 2002; Rautajoki et al., 2008; Seder and Paul, 1994).

All CD4 T cells leaving the thymus have a naive phenotype and require activation signals from DCs (Figure 1.5) in order to differentiate into effector T cells. The process of differentiation of a naive T helper (Th) cell into a Th1 or Th2 phenotype is regulated by many factors which includes T cell receptor (TCR) activation, co-stimulatory molecules on the surface of the antigen-presenting cell, and polarizing cytokines in the vicinity of the T cell (Figure 1.5). The cytokine environment has been shown to have a profound effect in generation of T helper cell subsets (Constant and Bottomly, 1997). IFN-γ and IL-12 are thought to be the major cytokines for promoting Th1 differentiation (Figure 1.5). IL-12 binds to the IL-12 receptor on naive T helper cells and triggers differentiation into Th1 cells by signaling through the STAT-4 signaling pathway (Rautajoki et al., 2008). IFN-γ is secreted mainly to prevent outgrowth of Th2 cells rather than promoting Th1 development. Besides IL-12, the other cytokines which play a role in Th1 differentiation include IL-18, IL-27 and IFN-α. Signaling through IL-4 receptor (IL-4R) is needed for adequate differentiation into the Th2 direction and proper Th2 responses. IL-4 activates STAT-6 protein, which then translocates to the nucleus to activate the transcription factor, GATA3, which then drives the expression of a broad array of Th2 cytokines (Murphy and Reiner, 2002). Once activated, Th2 cells can produce IL-4 in a STAT-6 independent manner.

Studies by Robinson and colleagues (Robinson et al., 1993a; Robinson et al., 1993b; Robinson et al., 1992) first described the evidence for activation of CD4+ Th2 cells in asthma patients as well as showed the contribution of CD4+ Th2 cells to asthma symptoms. The study by Robinson et al (Robinson et al., 1992) was the first to examine the cytokine expression profile in asthma patients. The study by Robinson and colleagues (Robinson et al., 1992) used 15 atopic asthma patients and bronchoscopy was performed in those patients to extract BAL samples. The BAL samples were then subjected to Giemsa staining and in situ hybridization studies for analyzing the cells and cytokine mRNA profile in the BAL samples. On analysis, results showed that a higher percentage of eosinophils and Th2-like cell populations were detected in the BAL samples from asthma patients along with increased expression of IL-4 and IL-5 mRNA from the BAL samples of asthma patients.

In another study by Robinson et al. (Robinson et al., 1993a), the identification of Th2 cells in the airways of asthmatic patients was first described. The study (Robinson et al., 1993a) used 15 atopic asthma patients who were either challenged with allergen or a diluent three times over a period of 9 weeks. Following 24 hours after allergen or a diluent challenge, BAL was extracted from those patients and analysed for presence of T cells and detection of cytokine mRNA. Flow cytometry analyses revealed a significant increase in activation of CD4+ T cells following allergen challenge while in situ hybridization results revealed a significant increase in expression of IL-4 and IL-5 mRNA. Immunomagnetic beads were then used to separate T cells from the BAL samples to examine whether Th2 cells are the source of the cytokine mRNA in the asthma patients. In situ hybridization was used to detect the cytokine mRNA from both the non-separated BAL sample and T cell-depleted BAL samples. Results revealed that both IL-4 and IL-5 mRNA was detectable in the non-separated BAL samples when compared to the T cell-depleted BAL samples which proved that Th2 cells are the source of cytokines in asthma patients. Hence, asthma is considered a Th2 cell-associated inflammatory disease, and Th2-type cytokines, such as IL-4, IL-5, IL-9 and IL-13 are thought to drive the disease pathology in patients.

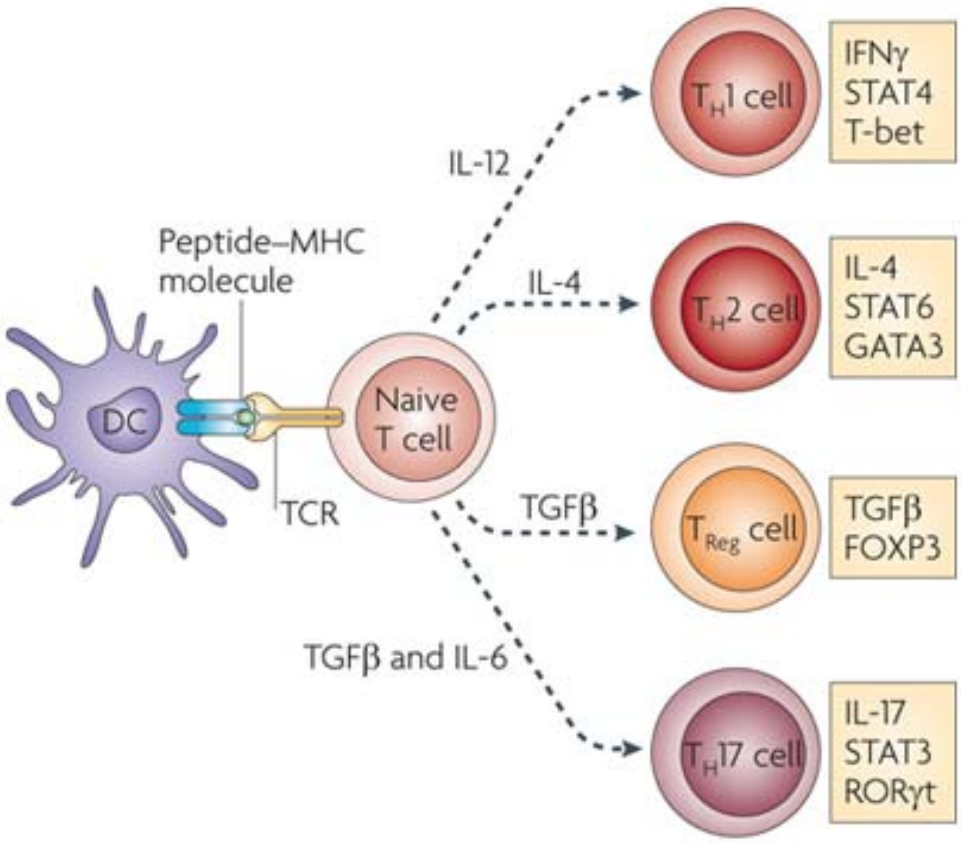

Figure 1.5: Overview of generation of the different T cell subsets by DCs. When naive T cells encounter antigen-laden DCs, they form a stable immunological synapse and T helper (Th) cell differentiation usually ensues, under the influence of instructive cytokines. Most of these cytokines are made by the dendritic cell itself, in the absence of any polarising stimulus for Th1, T regulating cell (Treg), or Th17 development. Generated T helper cell subsets have many functions in the immune system; some stimulate humoral immunity and others stimulate cellular immunity. FOXP3, forkhead box P3; GATA3, GATA-binding protein 3; IFNγ, interferon-γ; TCR, T cell receptor. Figure taken from (Zou and Restifo, 2010).

Although allergic asthma is a heterogeneous disease with allergen-specific Th2 cells playing a major role in asthma pathogenesis, this model is challenged by the recent discovery of Type 2 innate lymphoid cells (ILC2) that represent another critical innate source of type 2 cytokines. These ILC2 cells are activated by airway epithelial cell-derived cytokines, including IL-25, IL-33 and thymic stromal lymphopoietin (TSLP), which have been implicated in the initiation of asthma (Li and Hendriks, 2013).

Innate lymphoid cells (ILCs) are immune cells that lack a specific antigen receptor yet can produce an array of effector cytokines that in variety match that of T helper cell subsets. The term ILCs is collectively used to denote a variety of cell types that share common phenotypic and functional features (Spits and Cupedo, 2012). All ILCs have morphological characteristics of lymphoid cells yet lack rearranged antigen receptors, a quintessential feature of adaptive immune cells. ILCs react promptly to a wide array of signals and serve important roles in lymphoid tissue formation, repair of damaged tissue, and tissue homeostasis, as well as in immunity against infectious microorganisms. Several functionally distinct subsets of ILCs mediate different functions that can be grouped into three major categories (Table 1.1).

Table 1.1: The ILC family. Table taken from (Spits and Cupedo, 2012)

Cell type	Function	Signature cytokine produced by cells	Major stimulating cytokines
NK cells (cytotoxic ILCs)	Innate immunity against viral infections, tumor immunosurveillance	Interferon-γ	IL-18, IL-12, and IL-15
Rorγt$^+$ ILCs	Lymphoid tissue formation and repair, innate immunity against bacteria	IL-17 and IL-22	IL-1β and IL-23
Type 2 ILCs (ILC2)	Innate immunity against extracellular parasites	IL-5 and IL-13	IL-25 and IL-33

ILC2 require IL-7 for their development and produce T_h2 cell-associated cytokines in response to stimulation with the cytokines IL-25 (also known as IL-17E), IL-33 and TSLP (Spits et al., 2013). Unlike Th2 cells, these ILC2s are not antigen-restricted and are activated by epithelial cell-derived cytokines IL-25 and IL-33. The initiation of type 2 immune responses by the epithelial cell–derived cytokines IL-25, IL-33 and TSLP has been an area of extensive research in the past decade. Such studies have led to the identification of ILC2 cells as a critical source of type 2 cytokines *in vivo* which serve an important role in orchestrating the type 2 response to helminths and allergens (Licona-Limon et al., 2013).

The study by Fort and colleagues (Fort et al., 2001) first identified ILC2 cells in asthma murine models. The study by Fort and colleagues (Fort et al., 2001) found that intraperitoneal injection of IL-25 induced a Th2-like response, characterized by increased serum IgE, IgG1 and IgA, blood eosinophilia and pathological changes in the lungs and digestive tract. Their studies (Fort et al., 2001) identified Th2-type cytokine production by accessory cells which were subsequently identified as ILC2 in later studies by Moro and colleagues (Moro et al., 2010).

Interactions between ILC2 cells and the adaptive immune system, as well as examination of potential roles for ILC2 cells in the maintenance of homeostasis, promise to be particularly fruitful areas of future research. Further characterization of ILC2 cell biology will enhance the understanding of type 2 responses and may identify new treatments for asthma and allergies.

Objective, hypothesis and aims of the project

Objective

Understanding the pathogenesis of asthma by studying the lesional site in human asthma is limited by constraints related to tissue access, and they are usually restricted to sampling during periods when asthma is stable. Worst of all, the least understood phenotype in human asthma is the most severe form of the disease exemplified by acute severe exacerbations requiring hospitalization (Subrata et al., 2009b).

Data from murine studies suggests that the development of asthma, following respiratory infection with *Chlamydia* (Kaiko et al., 2008) results from engaging DCs which leads to recruitment of Th2 cells

in the lung resulting in subsequent asthma development. Translating these findings from murine models into human subjects is the primary objective of this project.

The presence of Th2-associated cytokines has been confirmed as a characteristic feature of the human asthmatic airway (Subrata et al., 2009b) and by assessing the cytokine responses of DCs, to *C. pneumoniae,* in patients with stable and acute exacerbations of asthma we aim to elucidate the role of this respiratory pathogen in asthma severity.

The project will utilize *in vitro* cultures of human monocyte-derived DCs (MoDCs) obtained from blood samples of healthy controls and asthma patients and infect them with *C. pneumoniae*. Following infection, *in vitro* cytokine analysis and gene array studies will be conducted on the infected DCs in order to determine the potential of infected MoDCs in driving a Th2 response which exacerbates asthma symptoms.

Hypothesis

We hypothesise that *C. pneumoniae* infection of MoDCs from asthma patients can exacerbate asthma by driving an enhanced immune response. By infecting MoDCs, from acute asthma and stable asthma patients, with *C. pneumoniae* we expect that MoDCs from asthma patients will respond with an increased immunostimulatory capacity which results in increased production of Th2 type mediators leading to asthma pathogenesis.

Aims of the project

1. Investigate the growth kinetics of *C. pneumoniae* in MoDCs and the effect of *C. pneumoniae* infection on MoDC maturation and viability

2. Determine the differences in surface maturation marker expression on MoDCs upon encountering *Chlamydia pneumoniae* in healthy controls, acute asthma and stable asthma patients.

3. Investigate different cytokine responses, to *Chlamydia pneumoniae*, between healthy controls and asthma patients during both stable periods and acute exacerbations

4. Investigate the differences in gene expression pattern on MoDCs upon encountering *Chlamydia pneumoniae* in healthy controls, stable asthmatics and acute asthmatics.

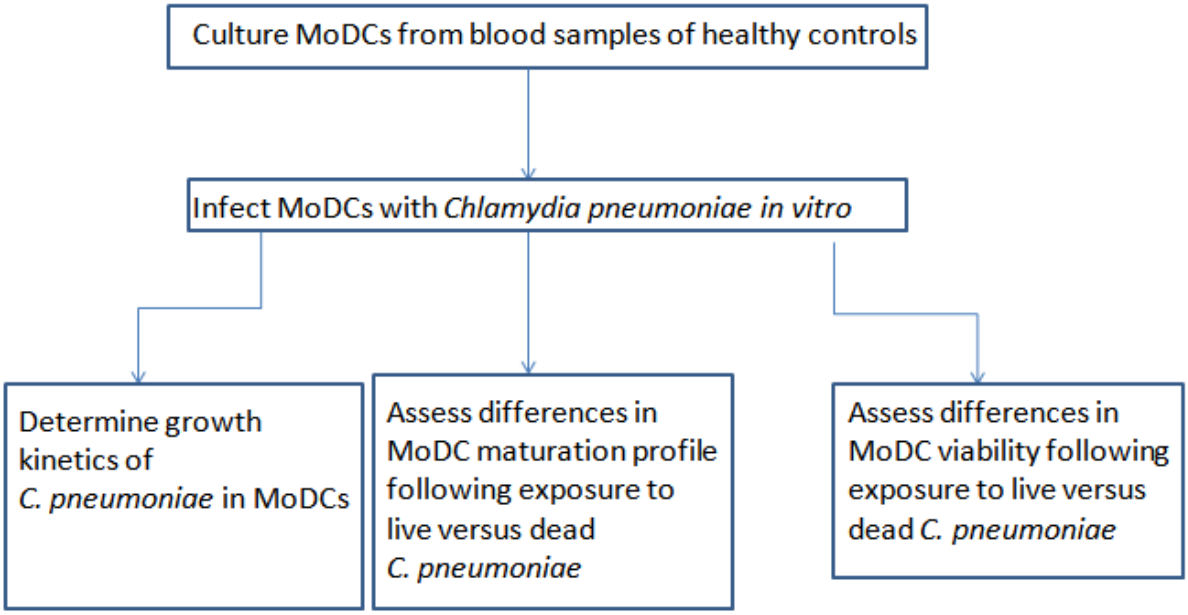

Figure 1.6: Overview of Aim 1. MoDCs, generated from healthy volunteers, will be infected with *C. pneumoniae* to deduce the ability of *C. pneumoniae* to replicate in MoDCs and assess the effect of infection on MoDC maturation and viability.

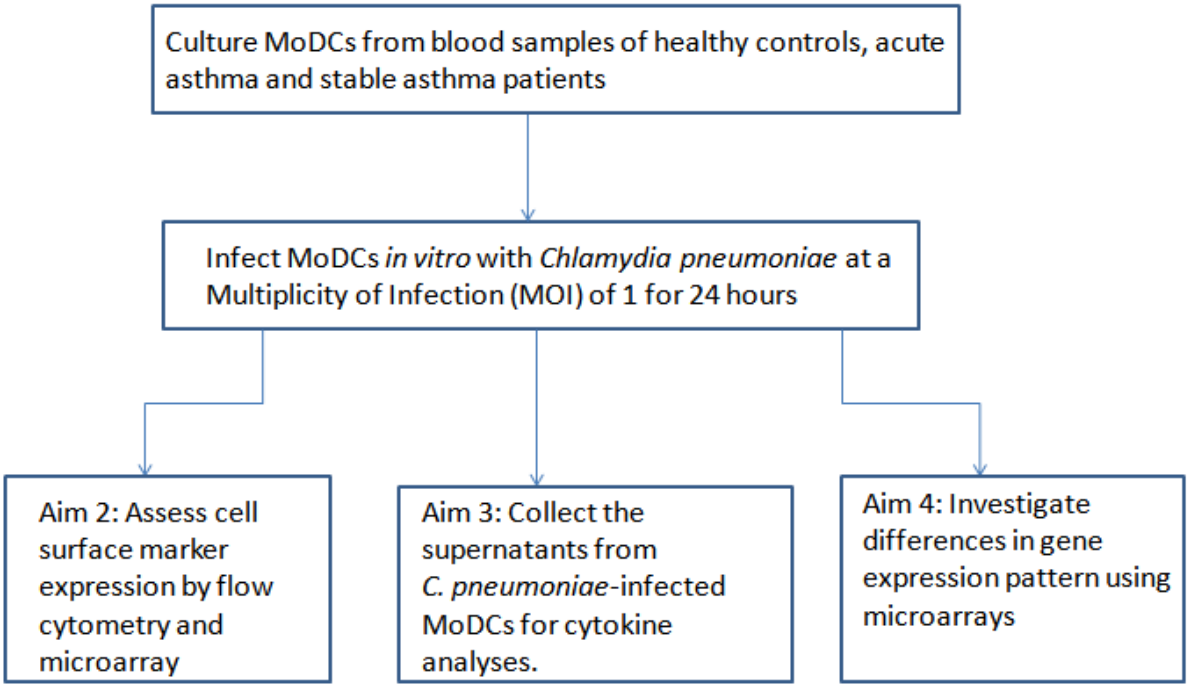

Figure 1.7: Overview of Aims 2, 3 and 4.

Significance and impact of this study

The potential role of respiratory infections as an initiating factor in asthma pathogenesis has been recognized for many years (Edwards et al., 2012). Multiple epidemiological studies (Awasthi et al., 2012; Biscione et al., 2004; Black et al., 2000; Gencay et al., 2001; Hahn et al., 1991; Hahn and McDonald, 1998; Hahn et al., 2006; Hahn et al., 2012; Johnston and Martin, 2005; Metz and Kraft,

2010; Patel et al., 2012; von Hertzen, 2002) have documented the presence of antibodies against *C. pneumoniae* infection in asthmatics which highlights the role of *C. pneumoniae* in asthma.

However, it is not yet clear on how exposure to *C. pneumoniae* can trigger asthma in humans. Hence, the ultimate goal of this project is to identify the pathways involved in the pathogenesis of bacterial induced asthma in humans. This will provide a more comprehensive understanding of the magnitude of the impact of these infections on asthma development.

Moreover, with our collaboration with respiratory physicians at Prince Charles Hospital (PCH), results from this study will contribute to an understanding of the mechanisms of a bacterial initiated Th2 response which could establish the groundwork for new therapeutic strategies to inhibit the development of asthma.

Chapter Two: Materials and Methods

Cultivation of cell lines, freezing & thawing of cells and cell counting

BEAS-2B cell culture

BEAS-2B (CRL-9609) human immortalized bronchial epithelial cells were a generous gift from Phillip Hansbro (University of Newcastle, Newcastle, Australia) and were maintained in Roswell Park Memorial Institute (RPMI 1640) supplemented with 2 mM L-glutamine, 100 µg/ml streptomycin, 0.1mM non-essential amino acids and 10% FCS (fetal calf serum). Human bronchial epithelial (BEAS-2B) cells were derived from normal bronchial epithelium obtained from autopsy of non-cancerous individuals. Cells were infected with a replication-defective SV40/adenovirus 12 hybrid and cloned (Reddel et al., 1988). For the purpose of propagating *C. pneumoniae* AR39 in cell culture, BEAS-2B cells were used.

Cell cryopreservation

For cryopreservation, cells were washed, resuspended in appropriate culture medium and cooled on ice. A cryopreservation solution of 10% dimethyl sulfoxide (DMSO; Sigma)/90% heat-inactivated FCS was prepared by slow addition of DMSO to the serum, followed by thorough mixing and cooling on ice. The cells were centrifuged and resuspended to a final concentration of $1x10^6$- $5x10^7$cells/ml by slowly adding an appropriate volume of the cryopreservation solution to the cell pellet with thorough mixing. The suspension was distributed into 1.2ml plastic cryogenic vials (Iwaki) and rapidly transported to a –70°C freezer in a 5100 Cryo 1°C freezing container (Nalgene/Thermo Fisher, Rochester, NY, USA) and 24h later to liquid nitrogen storage (–196°C).

Thawing of cells

Frozen cells were rapidly thawed in a 37°C water-bath and immediately diluted 1:10 in appropriate pre-warmed culture medium containing 20µg/ml DNase I (Roche). Following centrifugation at 500x*g* for 5min, supernatant was removed and cells resuspended in 1ml of appropriate medium prior to counting

Cell counting

Cell concentration was calculated using a haemocytometer (Boeco, Germany). In brief, the cells were resuspended and a sample was diluted in 0.4% trypan blue (Sigma). 10µl of the dilution was transferred to the haemocytometer counting chamber. Viable cells, as indicated by trypan blue exclusion, were counted using an Olympus BX40 light microscope (Olympus Corporation). Calculation of the cell concentration was performed using the following formula:

Cell concentration per ml = cells counted x dilution factor x 10,000.

Cultivation of *C. pneumoniae* AR39 culture and determination of Inclusion Forming Units (IFU)

Culture of *Chlamydia pneumoniae* AR39

Chlamydia pneumoniae AR39 (ATCC 53592) was used for infecting MOMoDCs in this project. AR39 is the first respiratory *C. pneumoniae* isolate which was obtained in 1983 from a university student with pharyngitis in Seattle, Washington, using HeLa 229 cell culture (Campbell and Kuo, 2009; Yan et al., 2008).

BEAS-2B cells were maintained in chlamydial culture medium [e.g., Roswell Park Memorial Institute (RPMI 1640) supplemented with 2 mM L-glutamine, 100 µg/ml streptomycin, 0.1mM non-essential amino acids and 10% fetal calf serum] and seeded in T-75 tissue culture flasks. A seed stock of *C. pneumonia*e AR39 was then inoculated onto the confluent monolayers of BEAS-2B cells, centrifuged at 3000 rpm for 30 min at 25°C and then incubated at 37°C for 2 h. Following incubation, chlamydial culture medium was removed and replaced with fresh chlamydial culture medium containing 1µg/ml cycloheximide and incubated for 72h at 37°C. *C. pneumonia*e was harvested by disruption of BEAS-2B cells with glass beads followed by sonication and centrifugation at 500g for 10 minutes at 4°C to remove cellular debris. Supernatants containing *C. pneumonia*e were centrifuged at 10,000 rpm for 30 min at 4°C to pellet *C. pneumoniae* elementary bodies (EBs). EB pellets were suspended in sucrose-phosphate glutamate (SPG) buffer, aliquoted and stored at -80°C.

Determination of the infectivity of the chlamydial stock

C. pneumoniae infectivity titers were assessed by an immunofluorescence (IF) assay. BEAS-2B cells were plated at $1X10^5$ cells/ml in 48-well tissue culture plates containing 10mm glass coverslips. Twenty four hours later, cells were then infected with serial dilutions of bacterial stock, cultured for 72hrs and then fixed with methanol. Coverslips containing infected cells were then washed with PBS and stained using the CelLabs Cel LPS staining kit, containing the fluorescein isothiocyanate (FITC)-labelled mouse monoclonal antibody specific for chlamydial lipopolysaccharide (LPS) (CelLabs, Brookvale, Australia), according to manufacturer's instructions. Stained coverslips are then placed on microscope slides and the FITC-stained chlamydial inclusions in BEAS-2B cells can then be visualized using Leica TCS SP5 confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany). Inclusions were observed using a Leitz laborlux fluorescence microscope (Leica Microsystems, Wetzlar, Germany). After counting for *C. pneumoniae* inclusions under a fluorescence microscope and correcting for dilution factors, bacterial titres were expressed as inclusion forming units (IFU/ml).

To calculate IFU/ml, the area of one 48-well (mm^2) at 40x magnification, was divided by the area of field examined (mm^2) at 40x magnification, giving fields/well. A minimum of 40 fields were counted, with the average multiplied by fields/well to calculate the number of chlamydial inclusions/well (IFU/well). Inoculum/well (µl) was calculated from the 1:2 dilutions performed and the inoculum/ml calculated. The inclusions/well (IFU) was multiplied by the inoculum/ml to calculate (IFU/ml).

Generation of monocyte-derived Dendritic cells (MoDCs) from subjects and *in vitro* infection with *C. pneumoniae*

Subjects

Adult volunteers (age>18 years old) were recruited into the study after undergoing clinical examination. Patients were recruited from the Prince Charles Hospital (PCH) and were divided into three groups. The groups included: healthy controls, stable asthma patients and acute asthma patients. A detailed clinical examination was performed by our collaborators in PCH, in order to diagnose the patients with asthma. The inclusion criteria for patients were as follows:

- Inclusion criteria for participants with stable asthma: Patients with a diagnosis of asthma defined as a clinical picture deemed compatible with asthma by a thoracic physician supported by demonstration of airflow reversibility with inhaled bronchodilators. Significant airflow reversibility is defined as an improvement in Forced Expiratory Volume at 1 second (FEV_1) of ≥ 12% or an increase of ≥ 200mls after nebulised salbutamol. Atopic asthma was defined as patients with a diagnosis of asthma who have an elevated IgE and a positive skin prick test for common allergens. Stability was defined as stable symptoms without need for oral steroids or unscheduled visits to their GP or hospital for asthma exacerbation symptoms. The history of the exacerbation, use of medications and lung function (if available) were recorded. Based on the patients history of asthma medications, patients were reported to be on interval medication such as an inhaled steroid and a sympathomimetic β2-agonist aerosol (bronchodilator - Ventolin, Bricanyl). Also use of a muscarinic receptor antagonist (Ipratropium, short-acting, or Tiotropium, long-acting), and preventive inhalation such as Cromones (Sodium Cromoglycate or Nedocromil) were reported in some of the patients. None of the stable asthma patients had a medication history of Omalizumab (IgE blocking antibody) or the leucotriene inhibitors.

- Inclusion criteria for participants with acute exacerbation of asthma: Patients with asthma were recruited from The Prince Charles Hospital Emergency Department at onset of presentation for treatment of an acute severe asthma exacerbation, defined as an acute worsening of asthma symptoms requiring systemic steroids (Hahn et al., 2006). The history of the exacerbation, use of medications and lung function (if available) were recorded. Peripheral blood was collected during an episode of acute asthma exacerbation and prior to treatment with corticosteroids in order to exclude any influence of corticosteroids on circulating blood.

- Inclusion criteria for healthy controls included no clinical history of asthma or any reported allergies

Blood collection was done by a nurse and sent to QUT for laboratory analysis. MoDCs were cultured from those blood samples and were used for the study. Human Ethics Approval Certificate has been obtained from Human Research Ethics Committee (HREC) of both PCH and QUT. The QUT human ethics approval number is 1100000225 and has clearance until 1st March, 2014.

Characteristics of study population

The study population comprised of a total of 55 subjects which were divided into three groups: healthy controls (n=13), acute asthma (n=15) and stable asthma (n=27) (Table 2.1). The study population was recruited as per criteria described above with blood collected from the patients and used to culture MoDCs for use in the project.

Table 2.1: Characteristics of subjects with asthma and healthy controls

	Healthy controls	Acute asthma	Stable asthma
Subjects (n)	13	15	27
Age (years)	56 ± 28	65 ± 20	63 ± 25
Male/Female	5/8	6/9	8/19
Asthma diagnosis	none	Recruited from the emergency department during an episode of asthma attack	Clinical history of asthma but can control the symptoms through use of medications

Generation of MoDCs from whole blood

Whole blood (24ml) was collected from subjects in vacuum tubes containing heparin and the blood was diluted 1:1 with PBS. Peripheral blood mononuclear cells (PBMCs) were isolated after density gradient centrifugation of the blood with Ficoll paque (GE Healthcare, Australia and New Zealand). Isolated PBMCs were subjected to positive selection using Monocyte Isolation Kit II (Mitenyi Biotec, Bergisch Gladbach, Germany) and purified using the AutoMACS system (Miltenyi Biotec) according to manufacturer's instructions. Purified CD14 monocytes were cultured for seven days in MoDC culture medium (RPMI 1640 supplemented with 2 mM L-glutamine, 100 µg/ml streptomycin, 0.1mM non-essential amino acids, 50µM 2-mercaptoethanol and 10% fetal calf serum) by plating in T25 tissue culture flasks at a density of $5\boldsymbol{X}10^5$ cells/ml of MoDC culture medium supplemented with 800 U/ml recombinant human GM-CSF (PeproTech, Rocky Hill, NJ, USA) and 1,000 U/ml of recombinant human IL-4 (Peprotech) (Tjhie et al., 1997). Following culturing for seven days, MoDCs generated from those blood samples were then harvested by pipetting the culture medium against the walls of the flasks as MoDCs are generally non adherent cells. MoDCs were analysed by flow cytometry to confirm the purity of MoDCs which involved staining MoDCs with pre-optimized amount of antibody against CD11c, CD14 & HLA-DR (Table 2.3) and analysing them on BD FACSAria™ III cell sorter (BD Biosciences). Morphology of MoDCs were also viewed under the light microscope where the distinct morphology of MoDCs can be characterized as large non-adherent cells floating around in the culture medium (Jones et al., 2012; O'Neill and Bhardwaj, 2005). For maturation, MoDCs were incubated with LPS (strain 0111:B4 Escherichia coli, Sigma-Aldrich) for 48 h. Mature MoDC served as positive controls for phenotyping of *C. pneumoniae*-infected MoDC.

Infection of MoDCs with *C. pneumoniae* AR39

Following generation of MoDCs from the cohort of subjects, immature MoDCs were harvested and infected *in vitro* with *C. pneumoniae* AR39. To prepare the chlamydial seed stock preparation for infection, 500µl MoDC culture medium was added to 500µl of a chlamydial seed stock preparation. The chlamydial suspension was diluted in sufficient MoDC culture medium to obtain the desired MOI (Multiplicities of infection). MOIs were calculated as the number of chlamydial IFU/number of human cells. Tissue culture plates were used for infecting MoDCs and plates were centrifuged at 700 × g for 45 min at 25° C followed by incubation at 37° C for 2 hours. Following incubation, only 500 µl of media was removed and replaced with fresh media which is followed by incubation for 24h at 37°C. Depending on the tissue culture plates used for plating MoDCs, an appropriate infection volume was chosen as mentioned in Table 2.2 to cover sufficiently the monolayers during the centrifugation process.

Table 2.2 Volumes used for *C. pneumoniae* infection of MoDC grown in different types of tissue culture dishes

Type of tissue culture plate	Infection volume (ml)
96-well plate	0.1
24-well plate	0.5
6-well plate	3

Infection of MoDCs with dead *C. pneumoniae*

MoDC maturation and viability in response to dead *C. pneumoniae* was examined using heat-killed and UV-killed *C. pneumoniae*. MoDCs were infected with dead *C. pneumoniae* in the same way as a live *C. pneumoniae* infection. Heat-inactivation of *C. pneumoniae* was carried out by heating to 70°C for 30 min (Sessa et al., 2009) while UV-inactivation of *C. pneumoniae* was carried out by exposure to UV light from a G15T8 UV lamp (D. William Fuller, Inc., Chicago, Ill.) at a distance of 5 cm for 45 min at room temperature as previously described (Lu et al., 2002). The IFU for both heat-killed and UV-killed *C. pneumoniae* were calculated from the titers determined on the original semi-purified *C. pneumoniae* seed stocks as described in methods. Inactivation of *C. pneumoniae* by UV and heat treatment was confirmed by the failure to develop inclusions following infection of BEAS-2B cells.

Analysis of *C. pneumoniae* growth by real-time PCR and growth curve

The aim of utilising 16S rRNA real-time PCR was to quantify the kinetics of chlamydial growth in infected MoDCs at different time points. Chlamydial DNA in the MoDCs can be measured by amplification of a sequence of the 16S rRNA gene (Wittkop et al., 2006) since 16S rRNA gene expression is often used as a measure of the growth rate and for the number of metabolically active *C. pneumoniae* (Polkinghorne et al., 2006). Moreover, *C. pneumoniae* 16S rRNA transcripts are known to be stably expressed in bacteria throughout the replication cycle (Shaw et al., 2000). BEAS-2B (bronchial epithelial cells) cells infected with *C. pneumoniae* served as positive controls.

BEAS-2B cells were plated at a density of $5X10^4$ cells per well in a 48-well plate and left overnight to grow to confluence. Cells were then infected with *C. pneumoniae* the following day with an MOI of 1.5 with total host cell and *C. pneumoniae* genomic DNA isolated from infected monolayers in 48-well plates at 24, 48, 60 and 72 hrs in triplicates. Similarly, MoDCs were plated at a density of $1X10^5$ cells/well in a 96-well flat-bottom plate and infected with *C. pneumoniae* with an MOI of 1.5 with total host cell and *C. pneumoniae* genomic DNA isolated from infected MoDCs at 24, 36, 48, 60 and 72 hrs in triplicates. Both BEAS-2B cells and MoDCs were infected with 150,000 IFU of *C. pneumoniae* AR39.

For isolation of genomic DNA from BEAS-2B cells and MoDCs, cell monolayers were trypsinised for two minutes, collected in 1.5ml eppendorf tubes and centrifuged at 500 g for 10 minutes at 4°C to pellet the cells. Following centrifugation, cells were washed twice with PBS and heat inactivated for 10 minutes at 95°C to kill the infectious intracellular *C. pneumoniae*. Genomic DNA was isolated and purified using the QIAmp DNA kit (Qiagen), according to the manufacturer's instructions. The 16S rRNA target gene was used to quantify the chlamydial DNA using primers qrt_16SF (5'-CTCAACCCCAAGTCAGCATT- 3') and qrt_16SR (5'-CTACGCATTTCACCGCT-ACA- 3') to detect an 86 bp region of the 16S rRNA gene (Mitchell et al., 2009). Real-time PCR reactions were performed in a final volume of 20 µl, including 0.05 µM each primer, 10 µl 2 X SYBR Green (Applied Biosystems, Foster City, CA), and 2.0 µl DNA. The thermal cycling conditions were 95°C for 7 min and repeat of 30 cycles of 94°C for 15 s, 61°C for 30 s and 72 °C for 30s. Samples were run in triplicates and all reactions were carried out using an Applied Biosystems 7300 real-time PCR system (Foster City, CA).

As a standard for *C. pneumoniae* 16S rRNA, a series of diluted *C. pneumoniae* DNA samples extracted from *C. pneumonia*e seed stock was used. Standards of known concentration of 10^2, 10^4, 10^6, 10^8 and 10^{10} copy numbers of the target 16S rRNA gene sequence were prepared as follows. DNA extracted from a sample of bacterial DNA, isolated from semi-purified stock of *C. pneumoniae* AR39 elementary bodies, was amplified by PCR using the 16S rRNA gene real time primers. The PCR product was electrophoresed in a 2% agarose/TBE (45 mM Trisborate and 1 mM EDTA, pH 8.0) gel, stained with ethidium bromide (0.5 µg/ml) and visualized with a UV transilluminator (λ = 302 nm). The band was cut out of the gel and the DNA purified using the High Pure PCR Product Purification Kit (Roche, Applied Science, Germany) according to the manufacturer's instructions. The concentration of DNA in the purified preparations was determined by spectrophotometric measurement of absorbance at 260 and 280 nm wavelengths. The number of molecules of product was calculated using Avogadroe's formula. Standards of known concentration (10^{10}, 10^8, 10^6, 10^4, 10^2 and 10^0) were prepared for the 16S rRNA target, and purified with a Wizard SV gel and PCR clean-up system according to manufacturer's instructions (Promega, USA). The relative concentration of *C. pneumoniae* 16S rRNA copies (number of copies per PCR) was calculated from the standard curve.

In order to assess the infectious progeny from MoDCs and bronchial epithelial cells following infection with *C. pneumoniae*, cells were harvested at 48h, 60h and 72h post infection and after infection and then disrupted by sonication (Sonic Dismembrator 60; Fisher Scientific, Pittsburgh, Pa.). The resulting cell lysates were serially diluted and inoculated onto fresh monolayers of BEAS-2B cells which were later stained with FITC-labelled mouse monoclonal antibody specific for chlamydial LPS (CelLabs, Brookvale, Australia). The chlamydial inclusion bodies were counted under a fluorescence microscope and were expressed as IFU/ml.

Flow cytometry

Phenotyping of MoDCs for surface markers

Exposure of MoDCs to microbial products induces the up-regulation of certain adhesion and costimulatory molecules which helps in antigen presentation and T-cell stimulation. These changes in expression of these cell surface proteins can be identified by means of flow cytometry which in turn can be used to determine the maturation state of MoDCs. Therefore, to analyse surface marker expression in human MoDCs following infection with *C. pneumoniae,* flow cytometry antibodies were used which included markers such as CD83, CD80, CD86 and HLA-DR (BD Biosciences: full details Table 2.3).

For phenotypic characterization of MoDCs, flow cytometry was used which involved staining MoDCs with fluorescently labelled antibodies and analysing them on BD FACSAria™ III cell sorter (BD Biosciences). Both uninfected and *C.pneumoniae*-infected MoDCs were washed and resuspended in PBS containing 5% Human AB serum (Sigma Aldrich) for thirty minutes at 4°C to prevent non-specific binding of antibodies to Fc receptors. To stain MoDC samples, cells were incubated for 25 min at 4°C in the dark with pre-optimized amounts of antibodies against CD80, CD83, CD86 and HLA-DR (BD Biosciences, USA: full details Table 2). Viability of MoDCs was determined by addition of Fixable viability stain 450 (BD Biosciences: full details Table 2.3) to identify non-viable cells during immunostaining. Following incubation, cells were washed and fixed with 4% paraformaldehyde (PFA) then re-suspended in FACS buffer (PBS containing 2% fetal calf serum), protected from light and stored at 4°C until flow cytometry analysis was performed. Cell acquisition was performed on a BD FACSAria™ III cell sorter (BD Biosciences) using BD FACSDiva™ software while analysis of flow data was performed using FlowJo software (Tree Star, Inc., Ashland, OR). Compensation was achieved using BD™ CompBeads (BD Biosciences).

Expression of cell surface marker was measured as Median fluorescence intensity. Median fluorescence intensity was chosen instead of mean fluorescence intensity (MFI) as it provided enhanced accuracy of measurement of MoDC cell surface markers. MFI is defined as the relative brightness of the fluorophore conjugate associated with the cell surface marker (Baumgarth and Roederer, 2000). Flow cytometry data are visualized using a single parameter histograms with the number of event (or cell counts) displayed on the y-axis while fluorescence intensity are represented on a logarithmic scale on the x-axis which generates peaks on the graph (Novo and Wood, 2008). This allows us to assess the levels of cell surface marker on MoDCs and generalize population of MoDCs expressing cell surface marker using either Median fluorescence intensity or mean fluorescence intensity (MFI). However, a skew in the histograms can either cause exaggeration or understate the MFI while Median fluorescence intensity is less influenced by skews on histograms. Hence, Median fluorescence intensity is considered a much more robust statistic which is less susceptible to significant shifts in histograms and was used for flow cytometry analysis.

LPS-treated mature MoDCs served as positive controls for phenotyping of *C. pneumoniae*-infected MoDCs. LPS was used as a positive control for inducing MoDC maturation because of its bacterial origin and its predominant role as a pathogen associated molecular pattern (PAMP). Moreover, LPS

represents a prototypical model of MoDC maturation as it can dramatically enhance expression of MoDC maturation markers. LPS (*Escherichia coli* serotype 0111:B4; Sigma-Aldrich, St Louis, MO) was supplied as lyophilized γ-irradiated powder, reconstituted in culture, and further diluted with DEPC water to working concentrations. To induce maturation in MoDCs, 200ng/ml LPS (strain 0111:B4 Escherichia coli, Sigma-Aldrich) was added to i MoDC cultures for 48hrs followed by staining with flow cytometry antibodies.

Detection of apoptosis

Annexin V staining was utilised to determine the effect of *C. pneumoniae* infection on inducing apoptosis in human MoDCs. One of the early commitment to apoptosis stages that occurs at the cellular membrane level is the extracellular exposure of phosphatidyl serine (PS) molecules on the cell membrane (Cohen et al., 1992). In normal viable cells, PS is located on the cytoplasmic surface of the cell membrane. However, in apoptotic cells, PS is translocated from the inner to the outer leaflet of the plasma membrane, thus exposing PS to the external cellular environment (Cohen et al., 1992) Annexin-V (Alexa flour 488) is a protein dye that has a strong affinity for phosphatidyl serine (PS) molecules in the presence of Ca^{2+}.

To detect apoptosis, both uninfected and infected MoDCs were first washed with cold PBS and with resuspended in 100µl annexin-binding buffer (10mM HEPES, 140mM NaCl, 2.5mM $CaCl_2$, pH7.4) followed by staining with 1µl Annexin V Alexa Fluor® 488 (AF488) (Invitrogen) antibody for 15 minutes at room temperature. Following Annexin V staining, cells were resuspended in 400µl of annexin-binding buffer and immediately analysed on a BD FACSAria™ III cell sorter (BD Biosciences) using BD FACSDiva™ software. Analysis of flow data was performed using FlowJo software (Tree Star, Inc., Ashland, OR) with apoptotic MoDCs quantified as percentage of cells stained positive for Annexin V Alexa Fluor® 488 (AF488) (Invitrogen). Heat-killed MoDCs were used as positive controls for apoptosis.

Table 2.3: Antibodies used for flow cytometry phenotyping

Antibody	Clone	Conjugation	Subclass	Final Dilution	Source
anti-human HLA-DR	G46-6	PE-Cy7	IgG2a	1:100 flow cytometry	BD Biosciences, San Diego, California, USA
anti-human CD11c	M5E2	FITC	IgG2a	1:100 flow cytometry	BD Biosciences, San Diego, California, USA
anti-human CD14	B-ly6	FITC	IgG1	1:50 flow cytometry	BD Biosciences, San Diego, California, USA
anti-human CD80	L307.4	PE	IgG1	1:100 flow cytometry	BD Biosciences, San Diego, California, USA
anti-human CD83	HB15	FITC	IgG1	1:50 flow cytometry	BD Biosciences, San Diego, California, USA
anti-human CD86	2331 (FUN-1)	APC	IgG1	1:50 flow cytometry	BD Biosciences, San Diego, California, USA
Fixable Viability Stain 450	-	BD Horizon™ V450	-	1:100 flow cytometry	BD Biosciences, San Diego, California, USA
IgG2a isotype control	G155-178	PE-Cy7	IgG2a	1:100 flow cytometry	BD Biosciences, San Diego, California, USA
IgG1 isotype control	MOPC-21	FITC	IgG1	1:50 flow cytometry	BD Biosciences, San Diego, California, USA
IgG2a isotype control	G155-178	FITC	IgG2a	1:50 flow cytometry	BD Biosciences, San Diego, California, USA
IgG1 isotype control	X40	PE	IgG1	1:100 flow cytometry	BD Biosciences, San Diego, California, USA
Annexin V	Purified Recombinant Annexin V	Alexa Fluor® 488	-	1:100 flow cytometry	Invitrogen

Cytometric bead array

MoDCs obtained from patients' blood samples were infected with *C. pneumoniae* for 24h with culture supernatant collected, centrifuged to remove any particulates and stored at -80°C until analysis. Levels of IL-1β, IL-6, IL-8, IL-10, IL-12p70 and TNF-α were measured using Cytometric Bead Array Human Inflammatory Cytokines Kit (BD Biosciences, USA) according to manufacturer's instructions. Meanwhile levels of IL-4 and IL-13 were determined by the cytometric bead array technology using human cytokine flex sets according to the manufacturer's instructions (BD

Biosciences, USA). Data was acquired using a BD FACSAria™ III cell sorter (BD Biosciences) and was analysed with FCAP analysis software (BD Biosciences).

Statistical analyses

Datasets are presented as means ± SEM (Standard Error of the Means). Paired t tests and Wilcoxon signed rank tests were performed to establish statistical significance between any two separate groups with different means. For Annexin V staining and MoDC viability studies, an ANOVA was used to analyse the difference between groups. A significant difference between any two groups was considered to exist when the $p<0.05$. Prism software version 5 was used for all calculations.

Genome-wide analysis of mRNA expression by Illumina microarrays

Study cohort for the microarray analysis

A sample cohort of fifteen patients was used for this part of the study. Five patients from each of the three groups' namely healthy controls, stable asthma and acute asthma were recruited from PCH as described above. The optimal number of patients required for the study was determined by statistical analysis with help from a biostatistician (Dr Dimitrios Vagenas, Research methods group, IHBI). Using statistical methods for sample size calculations (Whitley and Ball, 2002), the optimal number of patients required for each group was estimated to be five. Technical expertise from QFAB (Queensland Facility for Advanced Bioinformatics, The University of Queensland, Brisbane, Australia) was sought in analysing the microarray data from this project.

RNA extraction from human MoDCs

MoDCs obtained from patient blood samples were infected with *C. pneumoniae* AR39 for 24h and supernatant removed for cytokine analysis later. Total RNA was extracted by lysing cells using 500µl TRIzol reagent (Invitrogen, Australia) followed by purification using the RNeasy mini kit (Qiagen), according to the manufacturer's directions. After phase separation, the aqueous phase was further purified using the RNeasy mini kit (Qiagen) as per the manufacturer's instructions. Total RNA yield was measured by a NanoDrop™ Spectrophotometer whilst RNA purity was determined by measuring A260:A280 and A260:A230 ratios. Quantity and quality assessment using a NanoDrop™ Spectrophotometer is performed at multiple wave lengths at 240 nm (background absorption and possible contaminations), 260 nm (specific for nucleic acids), 280 nm (specific for proteins), and 320 nm (background absorption and possible contaminations) (Fleige and Pfaffl, 2006). An A260:A280 ratio between 1.9–2.1 and an A260:A230 ratio of 1.8–2.3 is generally considered an acceptable indicator of good RNA quality (Boeckx et al., 2011). The extracted RNA samples were sent to the University of Newcastle in Dr Nikola Bowden's lab for microarray studies.

Microarrays

Gene expression profiles were performed using the Illumina microarray platform. Human HT-12 v4 Expression BeaMoDChip (Illumina, San Diego, CA, USA) were used which contains a total of 47,231

probes covering 31,335 genes obtained from the National Center for Biotechnology Information Reference Sequence (NCBI Refseq) and Unigene databases.

For gene expression analysis, total RNA extracted from MoDCs was resuspended in DEPC water and sent to the University of Newcastle for microarray analysis. RNA samples (~1.2µg) were biotinylated and amplified by the Illumina TotalPrep RNA Amplification Kit (Ambion). cRNA products were hybridized to Illumina Human HT-12 v4 microarray chips. Fragmentation of cRNA, hybridization to Illumina microarrays, washing, staining and scanning was performed according to manufacturer's instructions (Illumina, San Diego, CA, USA)

Microarray data analysis

Raw microarray data were extracted and analysed using the open-source statistical computing language R (http://www.r-project.org) utilizing the Bioconductor package (Gentleman et al., 2004) called lumi where raw intensities were $\log_2$ transformed and quantile normalized. Differentially expressed genes were presented as gene expression ratios (expression level in *C. pneumoniae* AR39-infected MoDCs **divided** by the expression level in uninfected MoDCs) on the $\log_2$ scale to allow representation of biological comparisons containing $\log_2$ fold change.

Differentially expressed genes were then analysed using the limma package, where empirical Bayes was used to better estimate the variance of the genes. A linear model was fitted for each gene given a series of arrays using lmFit function. Unsupervised hierarchical clustering was performed using average linkage and Euclidean distance Using Benjamini and Hochberg method, the *P*-values were corrected for multiple testing (Bouaziz et al., 2012). Genes were defined as differentially expressed if the $\log_2$ fold change greater than 1 with p values ≤ 0.05.

Pathway Analysis

Functional annotations of the differentially expressed genes were identified using the GeneGo database by MetaCore™ (Wang et al., 2011) and Ingenuity Pathway Analysis (Ingenuity Systems®, www.ingenuity.com) softwares (Jimenez-Marin et al., 2009).

Probe sets, containing microarray data with $\log_2$ fold change, p-values & probe IDs, were uploaded into GeneGo®. Two separate GeneGo Enrichment Analysis (EA) procedures were performed on the gene lists. GeneGo defines an EA procedure as mapping gene IDs from the dataset onto gene IDs in entities of built-in functional ontologies (represented by canonical pathway maps, cellular process networks, disease biomarker networks, drug target networks, toxicity networks, and metabolic networks). Within each analysis the terms are statistically ranked based on their relevance within the dataset. First, a workflow for pair wise comparison between three groups was generated using GeneGo's "Compare Experiments" feature with thresholds of 1 and 0.05 for $\log_2$ fold change and p-value, respectively. This workflow resulted in the generation of bar charts comparing experimental data by analysing their intersections in terms of their mappings onto GeneGo's various ontologies, including Pathway Maps, process networks, Diseases, and GO processes. Second, a functional EA procedure was done using GeneGo's "Pathway Maps" feature which included datasets from all the three groups. This analysis gives a list of most significant pathway maps along with p-values.

Significant pathway maps from the workflow were then identified & investigated in terms of their biological relevance and p-values.

In GeneGo® the statistical significance (*p*-value) of the pathway maps are calculated using hypergeometric distribution. We defined the number of intersecting objects in the experiment as *r*, the number of network objects in the experiment as *n*, the total number of intersecting network objects in the database as *R*, and the total number of network objects in the database as *N*. A *p*-value was calculated for each object in the experiment based on its number of intersections (McEachin et al., 2008).

In order to obtain a reliable biological interpretation of the affected genes in our microarray experiment, microarray data were simultaneously examined using Ingenuity Systems Pathways Analysis (Ingenuity Systems®, www.ingenuity.com) software which categorizes identified genes based on biological function and signalling pathways. Probe sets, containing microarray data with $\log_2$ fold change, p-values & probe IDs, were uploaded into Ingenuity. Genes associated with a canonical pathway in the Ingenuity Knowledge Base were considered for the analysis. The significance of the association between the gene dataset and the canonical pathway was measured in two ways: (1) a ratio of the number of genes from the dataset that map to the pathway divided by the total number of molecules that exist in the canonical pathway; and (2) the Benjamini–Hochberg procedure for multiple testing correction, which allows us to calculate the false discovery rate for each of the probability values to determine whether the association between the genes in the dataset and the canonical pathway is explained by chance alone (Kaneko et al., 2013).

Chapter Three: Results

Aim 1:

C. pneumoniae infection of MoDCs: Growth kinetics and Maturation

Infection and viability of MoDCs

Infection and viability studies were undertaken to determine the specific MOI of *C. pneumoniae* needed to adequately infect 90-100% of MoDCs while maintaining viability of MoDCs. MoDCs were infected with an MOI of 0.5 and 1 as outlined in methods in Chapter 2. As observed in figure 3.1, confocal images revealed the presence of multiple bright chlamydial inclusions in majority of MoDCs when an MOI of 1 (panel B) is used while at an MOI of 0.5 (panel A) the intensity of positive *Chlamydia* staining is reduced. Hence, infection at an MOI of 1 can induce maximal infection of MoDCs.

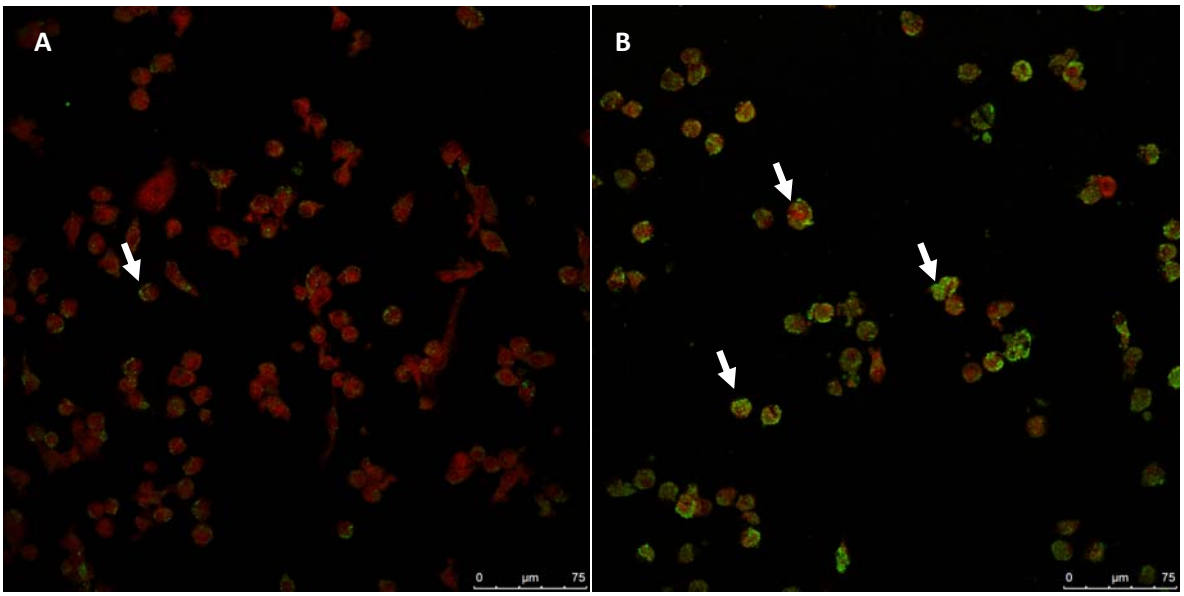

Figure 3.1: Confocal micrograph of human MoDCs infected with *C. pneumoniae* using immunofluorescence staining. MoDCs, generated by culture in GM-CSF and IL-4, were exposed to *C. pneumoniae* AR39 for 72h at an MOI of 0.5 (panel A) and 1 (panel B). Cell monolayers were stained with FITC-conjugated anti-*Chlamydia* LPS to detect the presence of inclusion bodies (green) within MoDCs counterstained with Evans blue (red). Scale bars represent 75µm taken under 100%. The white arrows indicate MoDCs infected with *C. pneumoniae*.

MoDCs play a role in inducing T-cell responses and their ability to survive a *C. pneumoniae* infection is important for induction of immune responses. Hence, viability of *C. pneumoniae*-infected MoDCs was investigated and whether increasing amount of *C. pneumoniae* can induce cell death in MoDCs. From figure 3.2, MoDCs exposed to increasing doses of *C. pneumoniae* underwent significant cell death ($p<0.001$) compared to uninfected MoDCs. *C. pneumoniae* induced cell death in MoDCs was not MOI-dependent (Figure 3.2) as *C. pneumoniae* infection at all MOIs resulted in 50% loss in cell viability.

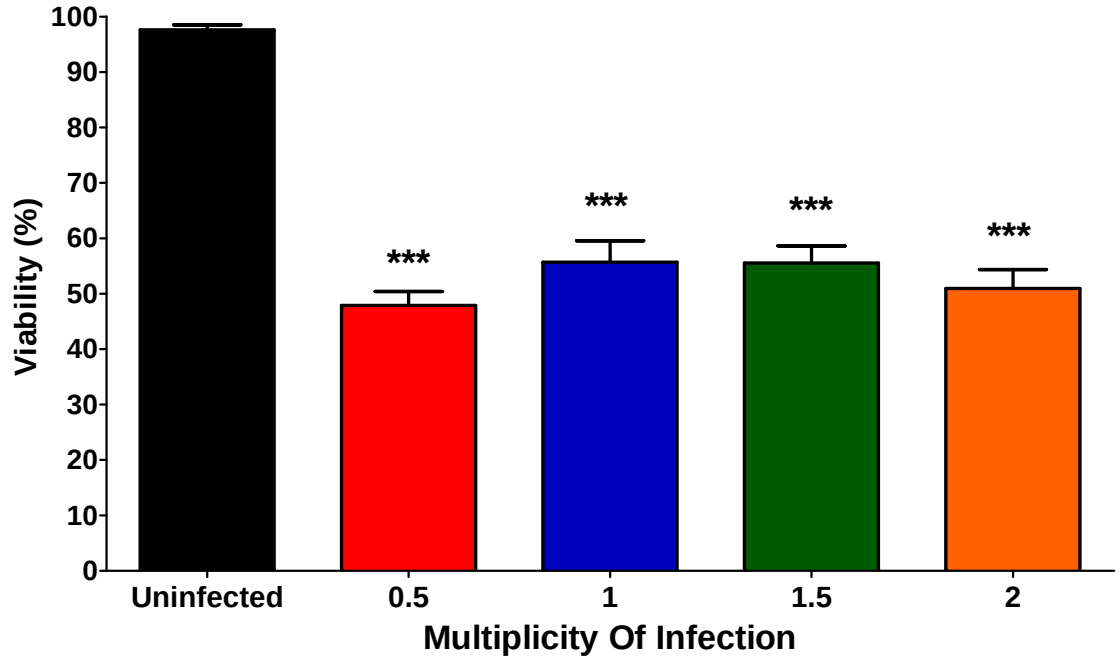

Figure 3.2: Viability of MoDCs with increased MOI of *C. pneumoniae*. MoDCs were infected *in vitro* with different given MOIs of *C. pneumoniae* for 24 hours. Viability of *C. pneumoniae*-infected MoDCs was determined by addition of Fixable viability stain 450 (BD Biosciences). Results indicated are an average of three independent experiments. Data represent mean ± SEM and were analysed by FlowJo software. Statistical analysis was done using one-way ANOVA (Tukey's multiple comparison tests). ***p<0.001 versus uninfected

C. pneumoniae replication in MoDCs and viable progeny

During normal chlamydial infection of epithelial cells, *C. pneumoniae* replicates inside epithelial cells to produce infectious progeny. In this study, bronchial epithelial cells were used as a positive control for monitoring intracellular replication in MoDCs. *C. pneumoniae* 16s rRNA gene transcripts were used to determine replication of *C. pneumoniae* in infected MoDCs and bronchial epithelial cells. As shown in figure 3.3 panel A, quantification of bacterial 16s rRNA transcripts revealed a significant ($p<0.05$) increase in chlamydial replication in bronchial epithelial cells. In contrast, no intracellular chlamydial replication in MoDCs was observed. This lack of chlamydial replication in MoDCs coincided with production of no infectious progeny at 72hrs post infection compared to bronchial epithelial cells (Figure 3.3, panel B).

These results demonstrate that *C. pneumoniae* can successfully infect MoDCs but they do not sustain replication and production of viable progeny.

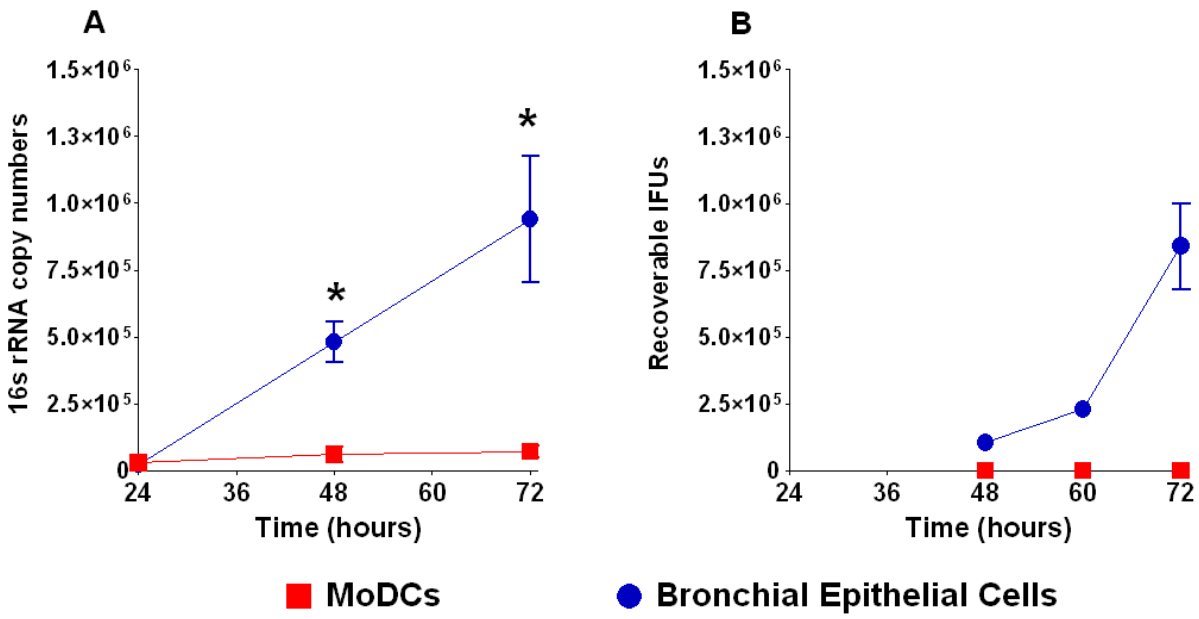

Figure 3.3: Panel A: Comparison of growth of *C. pneumoniae* in bronchial epithelial cells and MoDCs using 16sRNA real-time PCR. Both bronchial epithelial cells (blue line) and MoDCs (red line) were infected with *C. pneumoniae* at an MOI of 1.5. The data represent the means ± SEM (error bars) for three separate experiments, as shown in panel A, for each time point. Statistical analysis was done using two-tailed Mann-Whitney t test for each time point *p<0.05. Panel B: Growth of *C. pneumoniae* as reflected by recoverable IFUs. Both bronchial epithelial cells and MoDCs were infected with an MOI of 1.5 and sonicated at specified time points to extract cell homogenates which were later titrated onto fresh monolayers of bronchial epithelial cells to determine recoverable IFUs.

C. pneumoniae inclusion morphology in MoDCs compared with bronchial epithelial cells

Given the differences of *C. pneumoniae* to successfully replicate and produce infectious progeny between bronchial epithelial cells and MoDCs, the differences in morphology of *C. pneumoniae* inclusion bodies in bronchial epithelial cells and MoDCs was compared.

As shown in Figure 3.4, the inclusions display the characteristic pear shape of *C. pneumoniae* in bronchial epithelial cells (panel A). In contrast, *C. pneumoniae* inclusions in MoDCs did not form characteristic contained inclusion bodies and the bacerium was observed to be present throughout the cell (panel B).

This shows that *C. pneumoniae* can successfully infect MoDCs but with an altered morphology compared to epithelial cells.

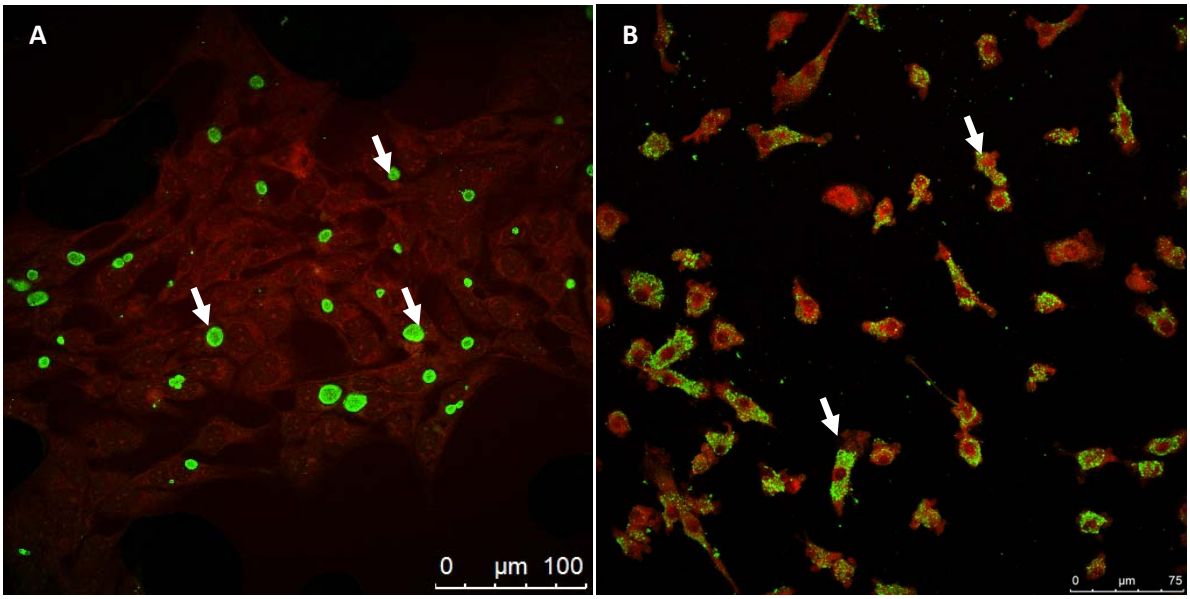

Figure 3.4: Comparison of inclusion bodies of *C. pneumoniae* AR39 in bronchial epithelial cells and MoDCs. Confocal micrograph of *C. pneumoniae* AR39 inclusions in bronchial epithelial cells (panel A) and MoDCs (panel B) cells when infected with *C. pneumoniae* at an MOI of 1 for 72hrs. Cell monolayers were stained with FITC-conjugated anti-*Chlamydia* LPS to detect the presence of inclusion bodies (green) within cells counterstained with Evans blue (red). Panel A shows *C. pneumoniae* infections in bronchial epithelial cells display the characteristic pear-shaped inclusions. Scale bars represent 100µm. Panel B shows *C. pneumoniae* infections in MoDCs did not form typical pear-shaped inclusions but present throughout the cell cytoplasm. Scale bars represent 75µm. Images were taken at a magnification of 20X using a Leitz laborlux S fluorescence microscope (Leica Microsystems, Wetzlar, Germany). The white arrows indicate *C. pneumoniae* inclusions which appear green while cells appear red.

MoDC maturation in response to *C. pneumoniae* infection

MoDC maturation is characterised by up-regulation of co-stimulatory molecules CD80, CD83, CD86 and HLA-DR. *Chlamydia* infection has been reported to alter DC maturation in mice (Kaiko et al., 2008) therefore the ability of live *C. pneumoniae* to alter surface expression of MoDCs maturation markers CD80, CD83, CD86, CD83 and HLA-DR was investigated by flow cytometry.

LPS (strain 0111:B4 *Escherichia coli*) was used as a positive control for inducing MoDC maturation. As shown in Figure 3.5, LPS induced a significant up-regulation of maturation markers CD80 ($p<0.01$), CD83 ($p<0.01$) CD86 ($p<0.05$) CD83 and HLA-DR ($p<0.01$) on MoDCs compare to unstimulated controls. In contrast, *C. pneumoniae* infection altered MoDC maturation profile. Of note, CD80 was significantly down-regulated compared to unstimulated controls ($p<0.01$) (Figure 3.5). Meanwhile, expression of other maturation markers CD83 and HLA-DR were significantly up-regulated by *C. pneumoniae* infection ($p<0.05$). Although slightly increased expression of CD86 was noted, it was not significantly altered compared to unstimulated MoDCs. This shows that *C. pneumoniae* infection in MoDCs leads to a distinct maturation profile characterised by selective down-regulation of maturation marker CD80.

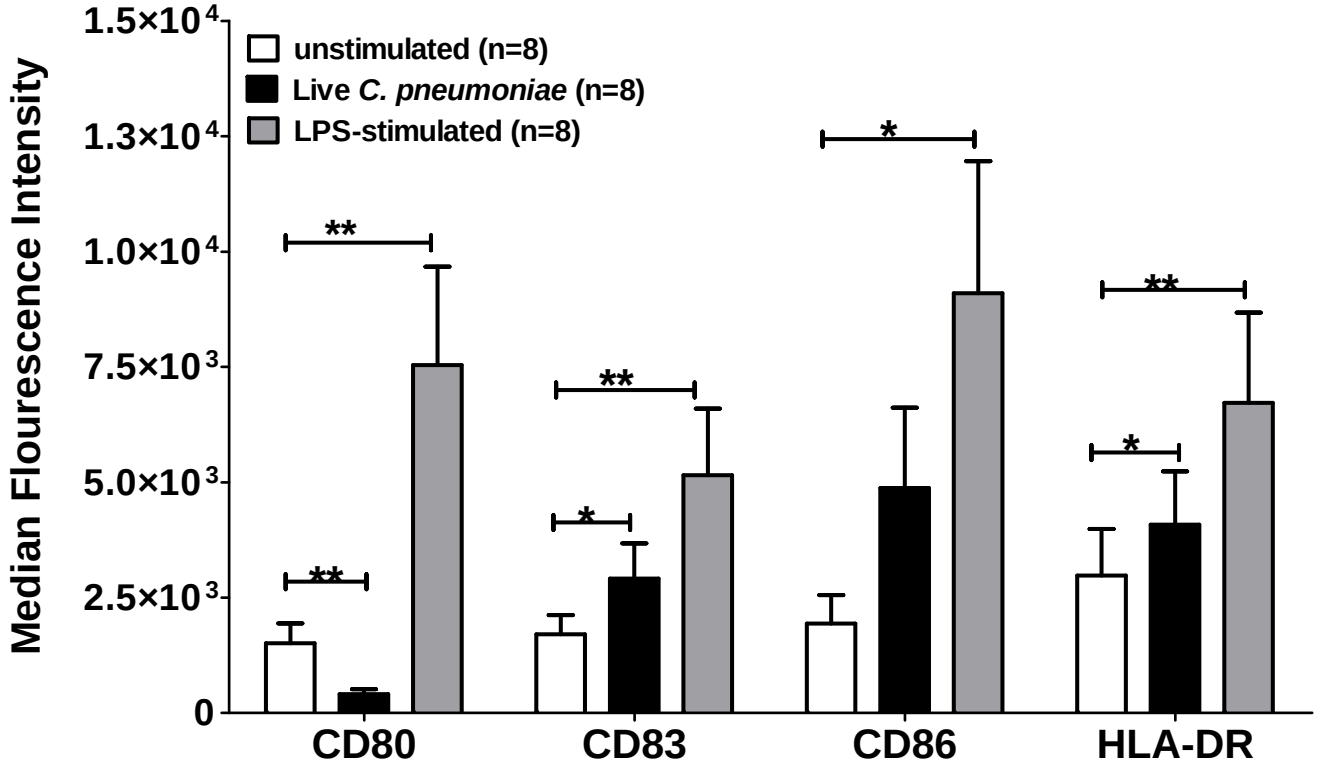

Figure 3.5: Surface expression of maturation markers CD80, CD83, CD86 AND HLA-DR on MoDCs. Maturation was determined 24 hours following infection with live *C. pneumoniae* (black bars, Live *C. pneumoniae*) or 200ng/ml LPS stimulation (grey bars) and compared to unstimulated controls (white bars). Data represent mean ± SEM from eight healthy controls (n=8) and analysed by Flowjo software. Statistical analysis was done using Wilcoxon matched paired t test. *p<0.05 versus unstimulated **p<0.01 versus unstimulated

MoDC viability and maturation in response to live versus killed *C. pneumoniae*

C. pneumoniae infection was observed to adversely affect viability of MoDCs. To determine if this is strictly dependent on infection by live *C. pneumoniae,* apoptosis, cell death and maturation was investigated in MoDCs following exposure to heat-killed and UV-killed *C. pneumoniae*.

The relationship between *C. pneumoniae* infection and viability of MoDCs was further investigated by exposing MoDCs to live, UV-killed and heat-killed *C. pneumoniae* using an MOI of 2 for 24 hours.

As shown in Figure 3.6, infection with live *C. pneumoniae* (red bars) significantly increased apoptosis (panel A) and cell death (panel B) ($p<0.001$) compared to uninfected controls. In contrast, MoDCs exposed to both UV- and heat-killed *C. pneumoniae* showed no significant difference in apoptosis or cells death compared to uninfected controls. Although a 2-fold increase in apoptosis was noted upon exposure of MoDCs to UV-inactivated *C. pneumoniae* (Figure 3.7, green bars), it did not reach statistical significance. This demonstrates that only live and not killed *C. pneumoniae* induces significant cell death and apoptosis in MoDCs.

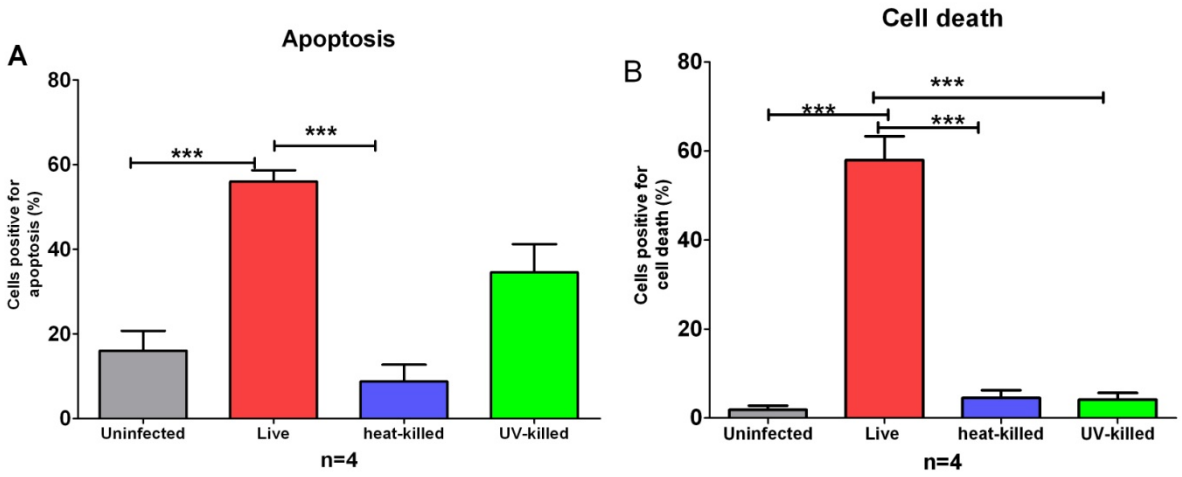

Figure 3.6: Relationship between *C. pneumoniae* exposure and induction of apoptosis and cell death in MoDCs. MoDCs, derived from four healthy volunteers, were exposed to live (red bars), UV-killed (green bars) or heat-killed (blue bars) *C. pneumoniae* for 24hrs at an MOI of 2. Panel A: For detection of apoptosis, MoDCs were stained with Annexin V Alexa Fluor® 488 (AF488) (Invitrogen). Panel B: Viability of MoDCs was determined by addition of Fixable viability stain 450 (BD Biosciences). Statistical analysis was performed using one-way ANOVA (Tukey's Multiple Comparison test). *p<0.05 **p<0.001

In contrast to apoptosis and cell death, UV-killed *C. pneumoniae* influenced the surface expression of maturation markers on MoDCs compared to uninfected controls. Figure 3.7 panel A showed that UV-killed but not heat-killed *C. pneumoniae* significantly enhanced CD80 expression ($p<0.05$) compared to uninfected controls. This directly opposes the effect of live *C. pneumoniae* in which decreased CD80 expression is observed (Figure 3.7, red bars, panel A). Interestingly, both live and UV-killed bacterial significantly enhanced CD83 expression ($P<0.05$) ($p<0.01$) (Figure 3.7, panel B). No significant difference in CD86 or HLA-DR expression was observed with both UV- and heat-killed bacteria compared to uninfected controls (Figure 3.7, panel C and D). In conclusion, these findings show that the selective down-regulation of CD80 on MoDCs is dependent on live and not killed *C. pneumoniea,* however UV-killed bacteria is immunostimulatory regulating the significantly increased expression of CD80 and CD83 along with non-significant increase in CD86 and HLA-DR.

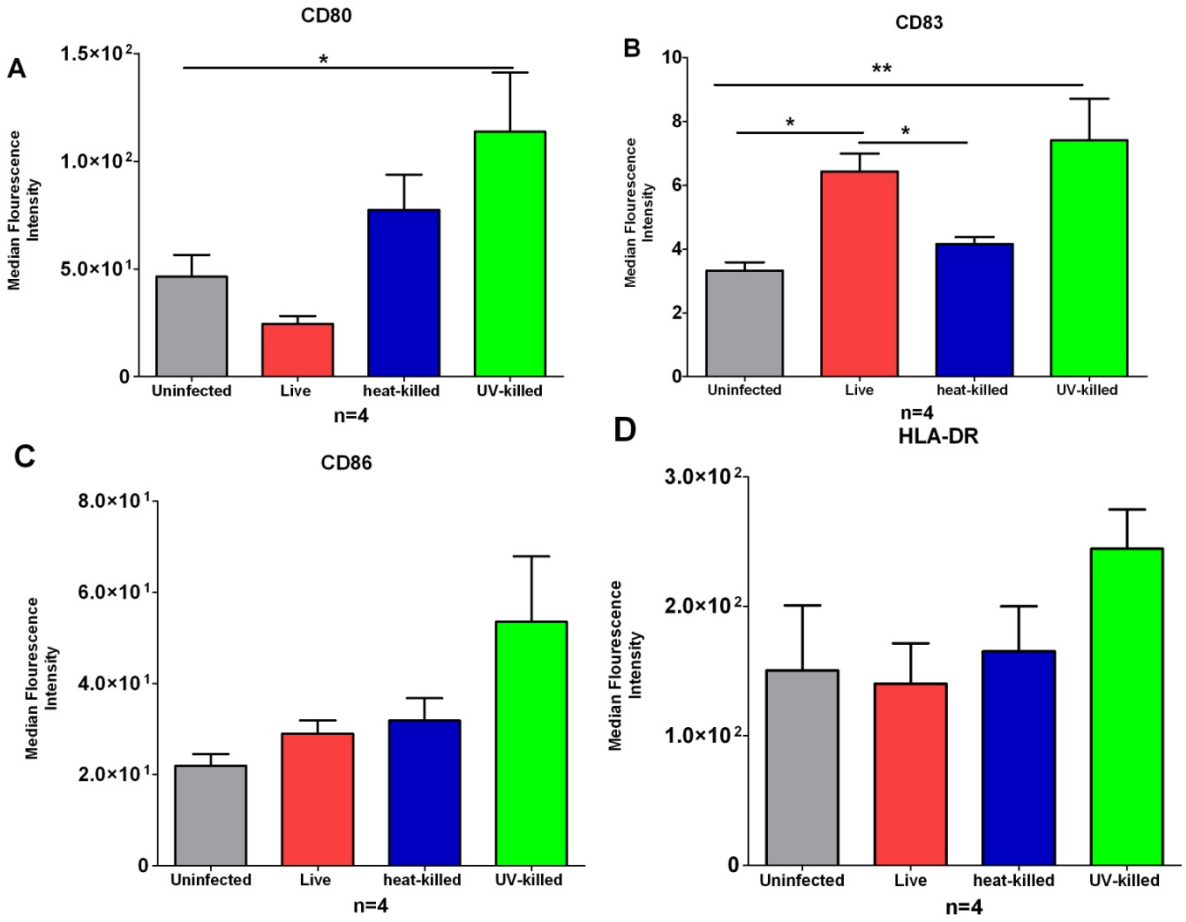

Figure 3.7: Distinct pattern of surface marker expression when MoDCs comes in contact with live (red bars), UV-killed (green bars) and heat-killed (blue bars) *C. pneumoniae*. MoDCs were exposed to live, UV-killed or heat-killed *C. pneumoniae* for 24hrs at an MOI of 2. The values indicated are the Median Fluorescence Intensity recorded on the cells stained with the specific antibodies to CD80 (panel A), CD83 (panel B), CD86 (panel C) and HLA-DR (panel D). Data represent mean ± SEM of four healthy volunteers analysed by FlowJo software. Statistical analysis was performed using one-way ANOVA (Tukey's multiple comparison tests) *p < 0.05 **p < 0.01

MoDCs responses to *C. pneumoniae* are not from cellular contamination of chlamydial stocks

Renografin purification of *Chlamydiae* is employed in chlamydial purification to remove cellular debris from lysed eukaryotic cells, as well as reticulate bodies, which are released with elementary bodies during sonication of infected cells (Campbell and Kuo, 2009). In this study the semi-purified stocks of *C. pneumoniae*, cultured from BEAS-2B cells, did not undergo density gradient centrifugation using renografin. As a result, unknown amounts of BEAS-2B cell debris have accumulated in the semi-purified stocks of *C. pneumoniae*. To deduce the effect of BEAS-2B cell debris on MoDCs maturation process, uninfected BEAS-2B cells were sonicated and purified in the same way as the chlamydial seed stocks as described in methods. MoDCs were exposed to the BEAS-2B cell debris, in the same way as *C. pneumoniae* infection, and then stained with DC maturation markers. As shown in Figure 3.8, exposure to BEAS-2B cell debris did not cause any change in surface expression markers on MoDCs which indicates the absence of any non-*chlamydia* related pathogenic stimuli in the semi-purified stocks of *C. pneumoniae*.

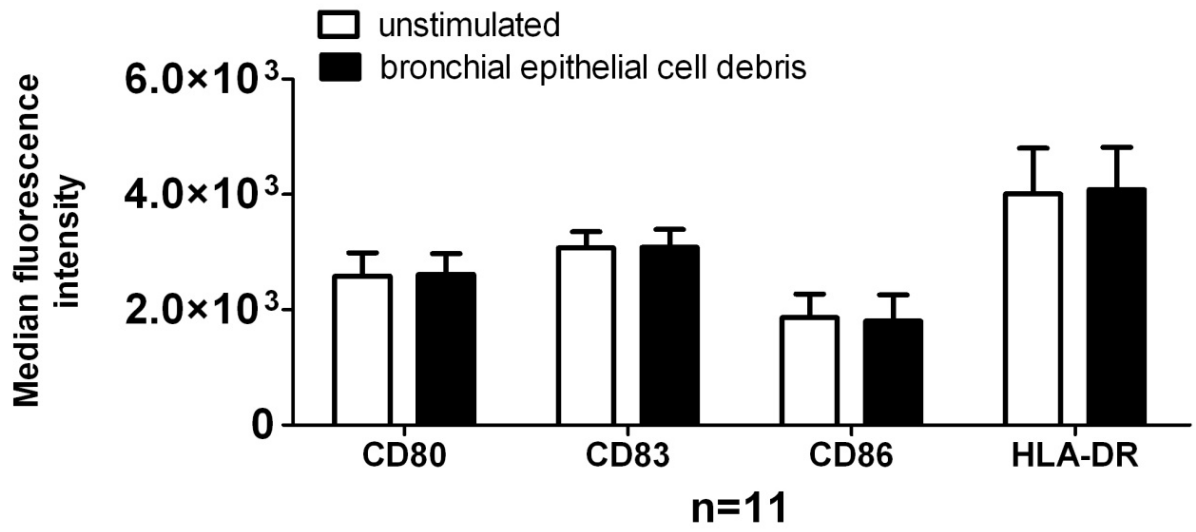

Figure 3.8: MoDCs stimulated with BEAS-2B cell debris. MoDCs, derived from eleven healthy volunteers, stimulated with BEAS2-B cell debris for 24 hrs and expression of CD80, CD83, CD86 and HLA-DR monitored before (white bars) and after addition of BEAS-2B cell debris (black bars). The values indicated are the Median Fluorescence Intensity recorded on the cells stained with the specific antibodies. Results indicated are an average of eleven independent experiments. Data represent mean ± SEM and were analysed by FlowJo software.

Aim 2:

MoDC maturation during *C. pneumoniae* infection in healthy controls versus asthma patients

Basal expression levels of maturation markers on MoDCs from healthy controls versus asthma patients

MoDCs were generated *in vitro* from CD14 monocytes, under the influence of cytokines GM-CSF and IL-4, as described earlier in the Chapter 2. Prior to infection with *C. pneumoniae*, the basal expression levels of maturation markers CD80, CD83, CD86 and HLA-DR were first evaluated in MoDCs generated from the patient cohorts. This will allow us to deduce whether asthma disease status had any effect on phenotypic commitment of CD14 monocytes into MoDCs.

As shown in Figure 3.9, MoDCs generated from the both acute and stable asthma cohorts displayed a significantly increased expression of maturation markers CD80, CD83, and CD86 ($p<0.05$) compared to healthy controls. No significant difference in expression of maturation markers was observed when asthma cohorts were compared. In contrast, expression of HLA-DR was significantly increased in MoDCs from the stable asthma cohort ($p<0.05$) but not in the acute asthma cohort compared to healthy controls (Figure 3.9). This shows that MoDCs derived from asthma cohorts differentiate into DCs with greater expression of co-stimulatory markers CD80, CD83 and CD86 (Figure 3.9) when compared to healthy controls. However, a key difference between MoDCs generated from the asthma cohorts is the low expression of HLA-DR on MoDCs from acute asthma patients while MoDCs from stable asthma patients display a significantly high expression of HLA-DR compared to healthy controls.

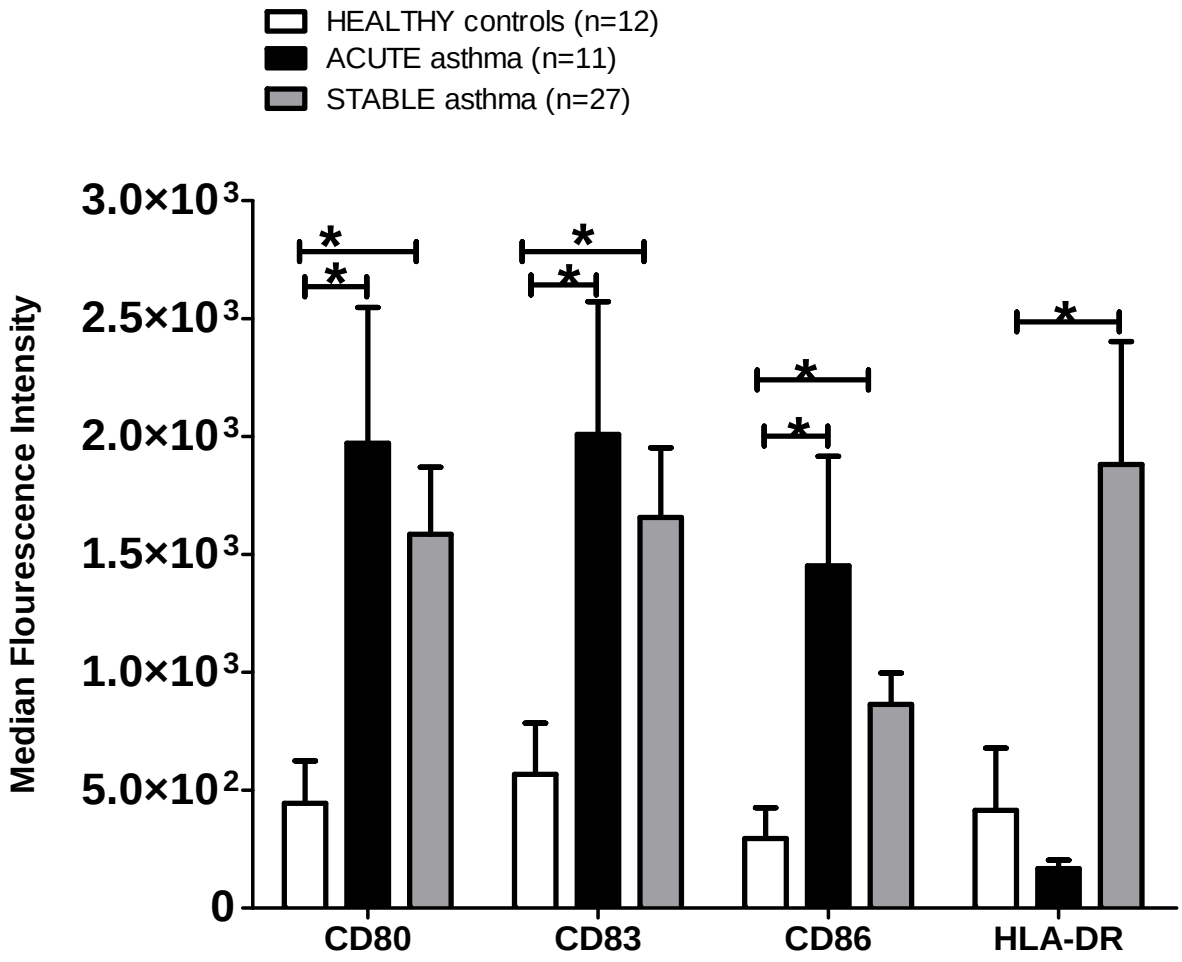

Figure 3.9: Surface marker expression on MoDCs generated from the patient cohorts. CD14 monocytes isolated from blood samples of the patient cohort were differentiated into MoDCs under the influence of cytokines GM-CSF and IL-4 for seven days. The values indicated are the Median Fluorescence Intensity recorded on the cells stained with the specific antibodies. Data represent mean ± SEM of 12 healthy, 11 stable asthma and 27 acute asthma individuals analysed by Flowjo software. Statistical analysis was done using two-tailed unpaired t test. *p<0.05 versus healthy controls

Effect of *C. pneumoniae* infection on MoDC maturation in healthy controls versus asthma cohorts

The effect of *C. pneumoniae* infection on MoDCs surface marker expression was assessed in each of the three patient cohorts, healthy controls (n=12), stable asthma patients (n=27) and acute asthma patients (n=11). MoDCs were infected *in vitro* with *C. pneumoniae* at an MOI of 1 for 24 hours and comparative surface marker expression on MoDCs was analysed via flow cytometry and RNA expression via microarray profiling. Microarray profiling of MoDCs maturation markers was done using a total of fifteen patients with five separate individuals from each of the patient groups: healthy controls, acute asthma and stable asthma.

From the flow cytometry data for CD80 in Figure 3.10 panel A, *C. pneumoniae* infection induced a significant down-regulation of CD80 in all the patient cohorts. In contrast, microarray profiling (Table 3.1) indicates an up-regulation of CD80 in response to *C. pneumoniae* infection in the patient cohorts (Table 3.1).

In response to *C. pneumoniae* infection, a notable difference in MoDCs phenotype was observed in the stable asthma cohort which is the significant up-regulation of MoDCs maturation marker CD83 seen specifically in the stable asthma cohort ($p<0.0001$) (Figure 3.10 panel B). Expression of CD83 from flow cytometry is consistent with microarray data (Table 3.1) as the stable asthma cohort displayed the 2.26-fold increase in CD83 expression when compared to other patient cohorts (Table 3.1).

From figure 3.10 panel C, no significant differences were observed in CD86 expression on MoDCs. In contrast, microarray profiling showed that CD86 expression was up-regulated in the asthma cohorts (Table 3.1) while flow cytometry data displayed no significant differences in the asthma cohorts (Figure 3.10).

From Figure 3.10 panel D, no significant differences were observed in HLA-DR expression on MoDCs. In contrast, microarray profiling (Table 3.1) indicates a down-regulation of HLA-DR in response to *C. pneumoniae* infection in the patient cohorts.

Collectively, these results suggest that *C. pneumoniae* infection leads to selective down-regulation of CD80, in all cohorts, along with a significant ($p<0.0001$) increase in CD83 expression in the stable asthma cohort. Meanwhile, CD86 expression on *C. pneumoniae*-infected MoDCs from asthma patients is shown to be modulated differently based on microarray profiling and flow cytometry analysis.

Table 3.1: Expression of MoDCs maturation markers in the patient cohorts determined by microarray gene expression analysis. Data presented are an average of five separate individuals from each of the patient groups: healthy controls, acute asthma and stable asthma. Numbers indicate fold change in expression of the genes in response to *C. pneumoniae* infection with negative numbers indicating down-regulation while positive numbers indicate up-regulation. "-" indicates not detected.

Gene Symbol	Gene name	Healthy controls	Acute asthma	Stable asthma
CD80	Cluster of Differentiation 80	1.33	2.22	2.7
CD83	Cluster of Differentiation 83	1.3	1.3	2.26
CD86	Cluster of Differentiation 86	-2.2	1.602	2.4
MHC II	Major Histocompatibility class II	-2.19	-2.7	-3.78
HLA-DRB4	Major histocompatibility complex, class II, DR beta 4	-2.82	-2.08	-2.06
HLA-DRB3	Major histocompatibility complex, class II, DR beta 3	-	-	-2.8
HLA-DRB1	Major histocompatibility complex, class II, DR beta 1	-	-	-3.3

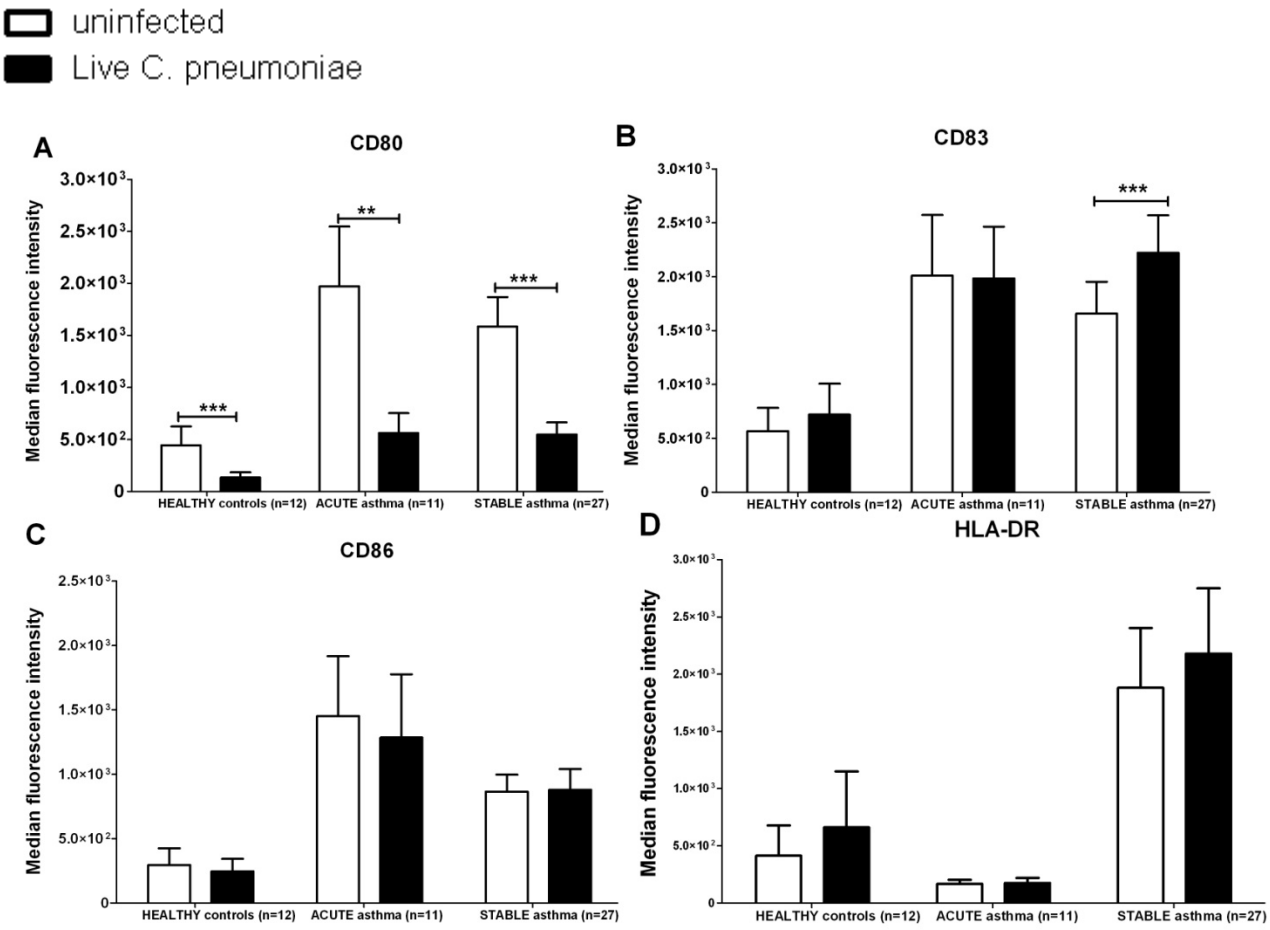

Figure 3.10: Comparative analysis of surface marker expression on MoDCs from healthy controls, acute asthma and stable asthma in response to *C. pneumoniae* infection. Surface marker expression was analysed by flow cytometry and expressed as median fluorescence intensity. Data represent mean ± SEM of 12 healthy, 11 acute asthmatic and 27 stable asthmatic cohorts analysed by FlowJo software. **p<0.01 and ***p < 0.001 versus uninfected controls using a two-tailed Wilcoxon matched paired t test.

Aim 3:

Cytokine secretion profile in response to *C. pneumoniae* infection in healthy controls versus asthma patients

Cytokines play an integral role in the coordination and persistence of the inflammatory process in asthma. Cytokine secretion by MoDCs was investigated using both microarray and *in vitro* cytokine analysis. For identification of significantly expressed cytokine genes from the microarray, a criterion of 2-fold ± expression and p<0.05 was applied. From the microarray profiling seven significantly expressed cytokine genes (Table 3.2) were identified which were further verified by *in vitro* cytokine analysis (Figure 3.11). As shown in table 3.2, stable asthma displayed the highest fold change in expression of cytokine genes compared to healthy controls while the acute asthma cohort and healthy controls displayed similar expression levels for cytokine genes (Table 3.2).

Expression of mRNA does not always correlate with protein expression. Hence, the cytokine gene expression pattern was further evaluated using *in vitro* cytokine analysis. Ten patients from each of the healthy controls, acute asthma and stable asthma group were used for *in vitro* cytokine analysis. MoDCs supernatants were collected and differential cytokine secretion profiles were assessed, as described in Chapter 2. IL12-p70 and IL-13 were undetectable in the MoDCs supernatants. As shown in Figure 3.11, *C. pneumoniae*-infected MoDCs from acute and stable asthma patients displayed a significant ($p<0.01$) increase in secretion of all the cytokines analysed which includes IL-1β (panel A), IL-4 (panel B), IL-6 (panel C), IL-8 (panel D), IL-10 (panel E), and TNF-α (panel F). Meanwhile, *C. pneumoniae*-infected MoDCs from healthy controls also displayed a significant increase in secretion of cytokines IL-1β (panel A), IL-4 (panel B), and TNF-α (panel F) along with non-significant increase in secretion of cytokines IL-6 (panel C), IL-8 (panel D) and IL-10 (panel E). Expressions of IL-4 gene was non-detectable in the microarray findings but were shown to be significantly secreted from *C. pneumoniae*-infected MoDCs in the patient cohorts ($p<0.01$) (Figure 3.11, panel B). From figure 3.11, *C. pneumoniae*-infected MoDCs from stable asthma patients displayed a comparatively increased secretion of all the cytokines compared to acute asthma cohort and healthy controls.

We also sought to identify other Th2-instructive cytokines like thymic stromal lymphopoietin (TSLP), IL-33 and IL-25 secretion (Hammad et al., 2009) by DCs using microarray. However expression of IL-33 and IL-25 was undetectable from the microarray profiling (Appendices, Supplementary Table 7) as it did not reach statistical significance. Meanwhile expression of TSLP was downregulated in healthy controls and upregulated by 0.5-fold in both stable and acute asthma patients (Appendices, Supplementary Table 7). Since the expression of TSLP gene did not reach the threshold criterion, we excluded it from further analysis. The threshold criterion for the gene expression data was defined as 2-fold ± expression and $p<0.05$.

Although expression of IL-12p70 was undetectable in supernatants derived from *C. pneumoniae*-infected MoDCs from our patient cohort (Figure 3.11), we further sought to investigate whether this can be attributed specifically to *C. pneumoniae*-infected MoDCs. As LPS is a well known inducer of IL-12 production from MoDCs (Jiang et al., 2002) we used LPS-stimulated MoDCs and *C. pneumoniae*-infected MoDCs, derived from two known healthy volunteers, to assess IL-12p70 production. Results (data not shown) revealed that IL-12p70 production was absent from *C. pneumoniae*-infected MoDCs while present in LPS-stimulated MoDCs which shows inhibition of IL-12p70 production in *C. pneumoniae*-infected MoDCs.

In conclusion, comparative cytokine analysis revealed that in response to *C. pneumoniae* infection, MoDCs from the asthma cohorts are characterised by a significantly increased expression of cytokines at both RNA (Table 3.2) and protein level (Figure 3.11) when compared to healthy controls.

Table 3.2: Expression of cytokine genes in the patient cohorts determined by microarray gene expression analysis. Data presented are an average of five separate individuals from each of the patient groups: healthy controls, acute asthma and stable asthma. Numbers indicate fold change in expression of the cytokine genes in response to *C. pneumoniae* infection with positive numbers indicate up-regulation. "-" indicates not detected.

Gene	Healthy controls	Acute asthma	Stable asthma
IL-1β	6.2	7.3	11.28
IL-4	-	-	-
IL-6	6.94	7.94	11
IL-8	-	7.64	11.8
IL-10	3.5	3.5	4.8
TNF-α	5.24	6	8.4
IL-12p35	-	-	2.7
IL-12p40	-	-	6
IL-23A (interleukin 23, alpha subunit p19)	7.78	8.32	13.26

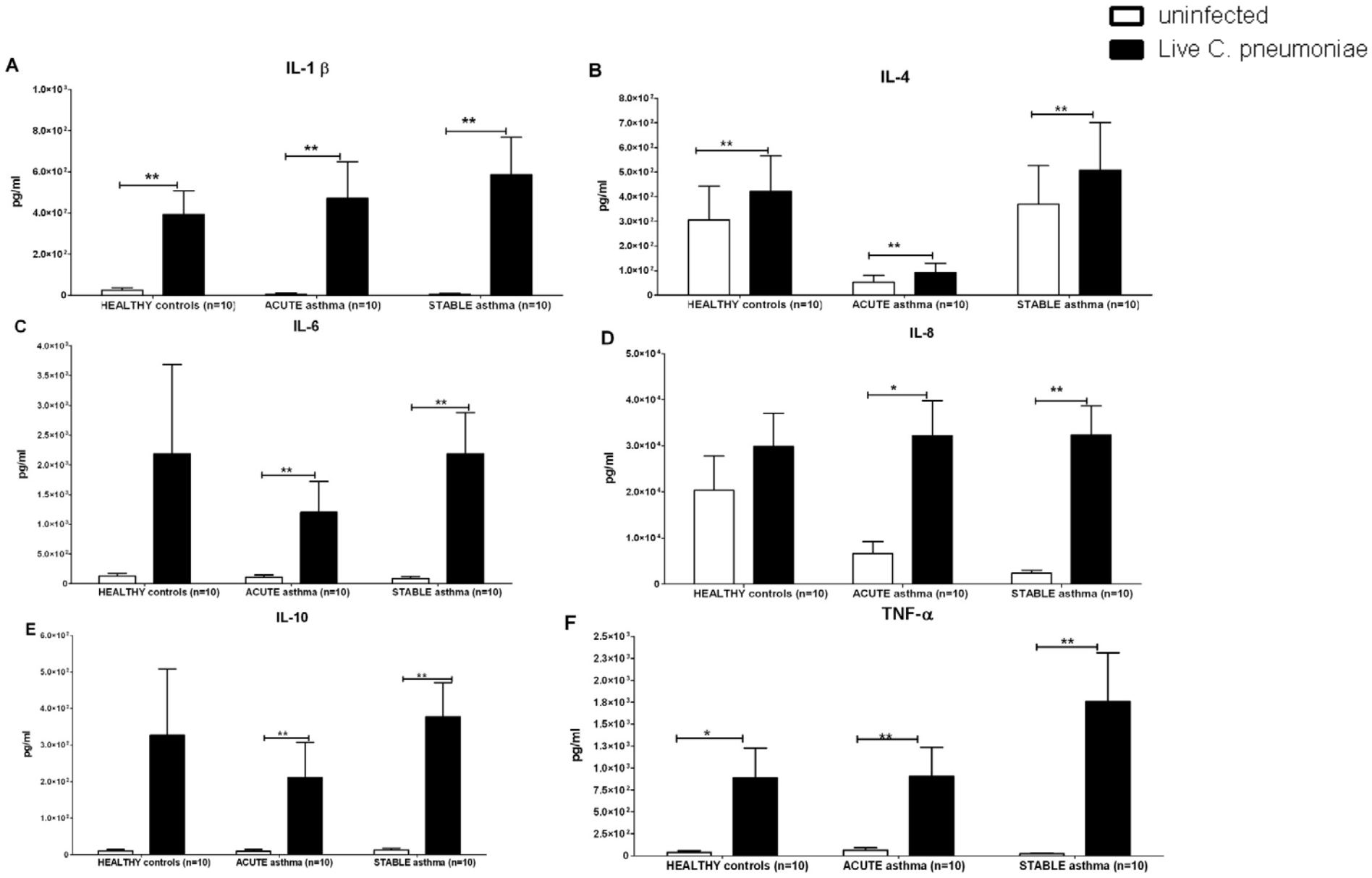

Figure 3.11: Cytokine production by DCs in the patient cohorts in response to *C. pneumoniae* infection. DCs isolated from the patient cohorts were infected *in vitro* with *C. pneumoniae* at an MOI of 1 for 24 hours with supernatant collected for cytokine analysis. No IL-12p70 and IL-13 were detectable in the *C. pneumoniae*-infected DCs. Data represent mean ± SEM. Statistical analysis was done using a two-tailed Wilcoxon matched paired t test. *p < 0.05 versus uninfectedp < 0.01 versus uninfected**

Aim 4:

Gene expression profiles in *C. pneumoniae*-infected MoDCs from healthy controls and asthma cohorts

In order to explore the impact of *C. pneumoniae* infection of MoDCs on gene expression profiles from the patient cohorts, microarray data from the Illumina arrays were analysed as described in Chapter 2. Differentially expressed genes were presented as gene expression ratios (expression level in *C. pneumoniae*-infected MoDCs **divided** by the expression level in uninfected MoDCs) on the $\log_2$ scale to allow representation of biological comparisons containing $\log_2$ fold change.

To visualise the differentially expressed genes for each groups, volcano plots (Figure 3. 12, 13 and 14) were generated. In this plot, the log2 expression ratio is plotted on the horizontal axis, whereas the statistical significance in shown on the vertical axis. To interpret the graph, the genes that are in the top right quadrants represent those that are most up-regulated while the genes that are in the top left quadrants represent those that are most down-regulated. For quantification of the number of genes differentially regulated in each plot, an arbitrary 2-fold cut-off and p-value less than 0.05 was established. This resulted in the list of 1975 genes differentially regulated in the healthy controls cohort (Figure 3.12), 1908 genes in the acute asthma cohort (Figure 3.13) and 3707 genes in the stable asthma cohort (Figure 3.14). Complete lists of differentially regulated genes resulting from microarray analysis are presented in the supplementary figures, which is available electronically.

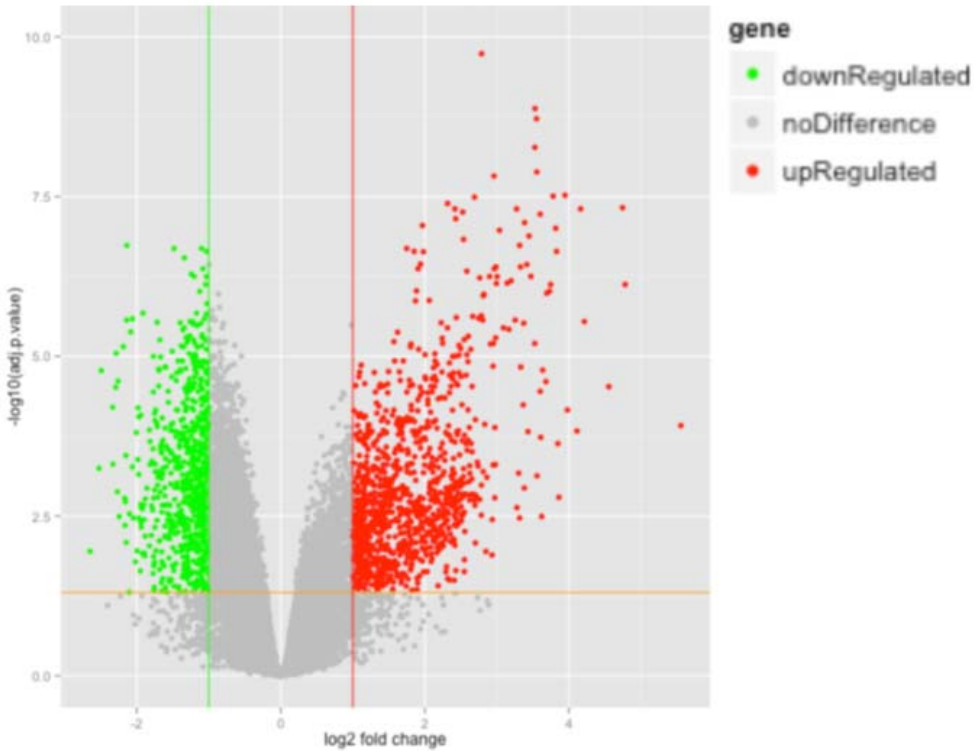

Figure 3.12: Microarray profiling of *C. pneumoniae*-infected MoDCs from healthy controls. Data presented as a volcano plot and is the average of five separate individuals. p<0.05 and log Fold Change=1. 1188 genes up-regulated and 787 genes down-regulated

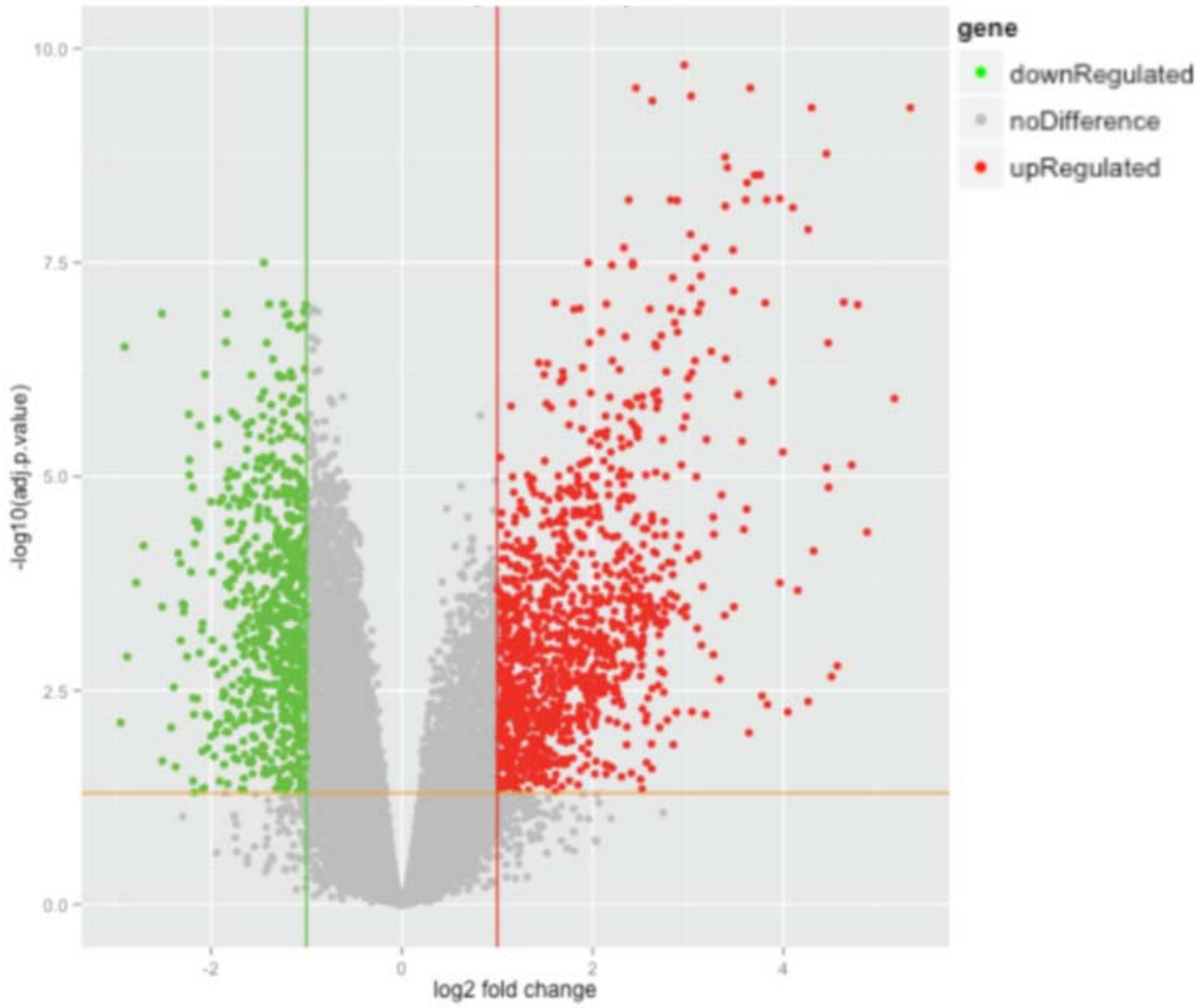

Figure 3.13: Microarray profiling of *C. pneumoniae*-infected MoDCs from acute asthma patients. Data presented as a volcano plot and is the average of five separate individuals. p <0.05 and log Fold Change=1. 1208 genes up-regulated and 700 genes down-regulated

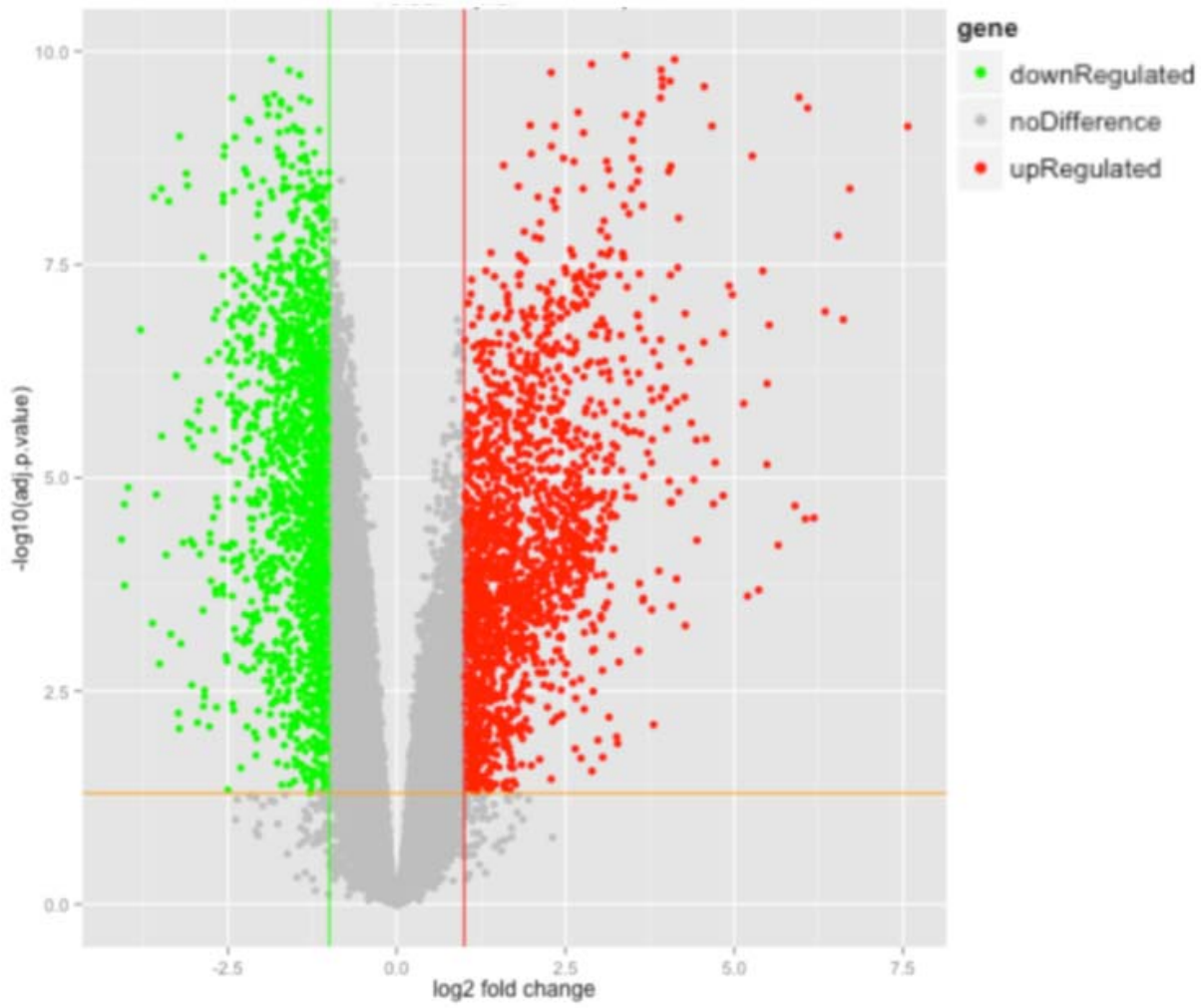

Figure 3.14: Microarray profiling of *C. pneumoniae*-infected MoDCs from stable asthma patients. Data presented as a volcano plot and is the average of five separate individuals. p<0.05 and log Fold Change=1. 1943 genes up-regulated and 1764 genes down-regulated

Using the list of differentially expressed genes from the volcano plots (Figure 3.12, 3.13 and 3.14), a Venn diagram (Figure 3.15) was constructed which enabled us to quantify the overlap of genes in response to *C. pneumoniae* infection in the patient cohorts. For each of the three groups, genes with a differential regulation of at least ± 2-fold changes ($p<0.05$) were considered statistically significant and analysed in a Venn diagram. As shown in Figure 3.15, a total of 1337 genes are commonly shared between the entire patient cohorts. Meanwhile, 314 genes are shown to be solely expressed in the healthy controls (Figure 3. 15) which are termed as "unique genes". The number of "unique genes" in the stable asthma and acute asthma cohort stood at 1615 and 65 respectively.

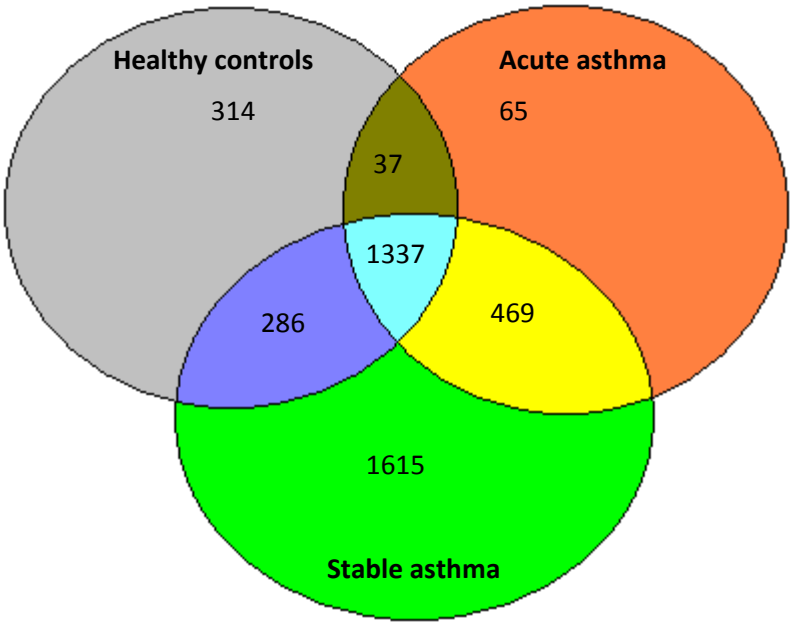

Figure 3.15: Venn diagram showing the overlap between differentially expressed genes among healthy controls, acute asthma and stable asthma.

Biological pathway analysis

A benefit of microarray analysis is that it can be used to discover associated genes that were previously thought to be unrelated to immune responses against *C. pneumoniae*. On the other hand, it was a daunting challenge to interpret complex biological processes from such a long list of genes, as there were significant overlaps in biological processes between the three patient cohorts. To deal with these difficulties, we compared the lists of differentially expressed genes relative to healthy controls to determine the degree of overlap and divergence among asthma cohort using GeneGo's Compare Experiments Workflow. These generated bar charts (Figure 3.16) which shows the number of genes that are common, similar and unique between two given data sets.

From figure 3.16, a pair wise comparison of microarray datasets between healthy controls and stable asthma (panel A) revealed that 1562 genes are commonly shared while 249 genes are unique only to healthy controls and 1762 genes are unique only to stable asthma. A pair wise comparison of microarray datasets between healthy controls and acute asthma (panel B) revealed that 1324 genes are commonly shared while 491 genes are unique only to healthy controls and 430 genes are unique only to acute asthma. A pair wise comparison of microarray datasets between acute asthma and stable asthma (panel C) revealed that 1665 genes are commonly shared while 72 genes are unique only to acute asthma and 1649 genes are unique only to stable asthma. Notably, the stable asthma group had many genes that were unique indicating that *C. pneumoniae* infection had a profound effect in gene expression profiles of these MoDCs. This allowed prediction of unique, shared and differential effects of *C. pneumoniae* infection in the patient cohorts.

A

#	Color	Experiment name	Species
1		healthy controls	Homo sapiens
2		stable asthma	Homo sapiens

B

#	Color	Experiment name	Species
1		healthy controls	Homo sapiens
2		acute asthma	Homo sapiens

C

#	Color	Experiment name	Species
1		acute asthma	Homo sapiens
2		stable asthma	Homo sapiens

Figure 3.16: Distribution and number of unique, similar and common features between the data sets. Common genes - striped, similar – white and unique – coloured bars. Microarray datasets were entered into GeneGo's Compare Experiments Workflow using a threshold of p<0.05 and fold change ± 2. Panel A shows comparison between healthy controls and stable asthma while panel B shows comparison between healthy controls and acute asthma. Panel C shows comparisons between acute asthma and stable asthma.

To gain an appreciation of the various functions associated with these differentially expressed genes in the patient cohort, the software packages GeneGo and Ingenuity were utilized. An enrichment analysis done using the Ingenuity software to identify the expression of various immune response genes shown in Tables 3.3, 3.4, 3.5, 3.6 and 3.7

Table 3.3: Expression of commonly shared immune response genes in healthy controls and the asthma cohorts. Values represent gene expression ratios (expression level in *C. pneumoniae*-infected MoDCs divided by the expression level in uninfected MoDCs) on the log_2 scale

Gene Symbol	Gene name	Healthy controls	Acute asthma	Stable asthma
AP-1	Activator protein-1	3.56	3.97	4.66
c-Jun/c-Fos	c-Jun/c-Fos Complex	2.03	1.82	2.24
CREB-1	cAMP responsive element binding protein 1	2.17	2	1.98
HSP70	Heat shock protein 70	3.09	3.52	3.58
I-κB kinase	Inhibitory kappa B kinase	1.71	1.26	1.6
NF-KB	NF-kB Group of complexes	1.73	1.81	2.45
p21	cyclin-dependent kinase inhibitor 1	1.11	1.11	1.64

As shown in Table 3.3, genes encoding for inflammatory transcription factors AP-1, CREB-1 and NF-KB were up-regulated at similar levels in all the patient cohorts in response to *C. pneumoniae* infection which suggests induction of innate inflammatory responses are shared equally among the patient cohorts. The AP-1 transcription factor is a dimeric complex that comprises members of the c-Jun/c-Fos Complex, ATF (activating transcription factor) and MAF (musculoaponeurotic fibrosarcoma) protein families. The AP-1 complex can therefore form many different combinations of heterodimers and homo-dimers, and this combination determines the genes that are regulated by AP-1 (Uronen-Hansson et al., 2004). Expression of these inflammatory transcription factors lead to coordinated expression of multiple inflammatory cytokines and chemokines as shown in Tables 3.4 and 3.5. Apart from transcription factors, other commonly shared immune response genes included HSP70 and p21 are shown to be expressed at equal levels in all the patient cohorts in response to *C. pneumoniae* infection. Given the role of TLR2, TLR4 (Flego et al., 2012; Prebeck et al., 2001), Nucleotide-binding oligomerization domain-containing protein 1 (NOD1) and Nucleotide-binding oligomerization domain-containing protein 2 (NOD2) (Shimada et al., 2012) in detection of *C. pneumoniae* in immune cells, we explored them in the array data. However, they were all shown to be downregulated (Appendices, Supplementary Table 7).

Asthma-specific gene expression profiles

To determine significant gene expression changes in the patient cohorts, a threshold criterion was applied to compare the differences in gene expression profiles from *C. pneumoniae*-infected MoDCs in the patient cohorts. The threshold criterion was defined as 2-fold ± expression (1 on the log_2 scale) and $p<0.05$. Using this criterion, several significantly expressed genes were identified with higher expression in the asthma cohorts (Table 3.4 and 3.5) and selectively expressed in the asthma cohorts (Table 3.6) compared to healthy controls. These differentially expressed genes can be grouped into three different classes of genes: chemokines, colony-stimulating factors (CSFs) and airway remodelling genes.

i. Chemokine gene expression profiles in healthy controls versus asthma cohorts:

Chemotactic cytokines or Chemokines are a family of small proteins that signal through G-protein–coupled receptors and are classically associated with immune cell recruitment to sites of injury. As shown in Table 3.4, nine different chemokine genes were identified which were significantly expressed in asthma patients using the thereshold criterion.

Eosinophil attracting chemokine genes, CCL1, CCL3, CCL4 and CCL5, are up-regulated in the stable asthma patients by 1.6-2-fold when compared to healthy controls. Meanwhile, expression of these eosinophil attracting chemokine genes are up-regulated in the acute asthma cohort by 1.3-2.2-fold when compared to healthy controls.

CCL20 expression is increased by 1.6-fold in the stable asthma cohort compared to the healthy controls. CCL20 acts as a DC chemoattractant which can mediate the accumulation of immature DCs within the lungs (Osterholzer et al., 2005).

CXCL1, CXCL2 and CXCL5 are defined as neutrophil attracting chemokines based on their presence in bronchial biopsies of COPD patients with an increased neutrophilia (Qiu et al., 2003). These chemokines plays a crucial role in recruiting neutrophils in response to microbial infection and tissue injury. As shown in Table 3.4, expression of neutrophil attracting chemokines CXCL1, CXCL2 and CXCL5 are up-regulated by 1.3-1.9-fold in the stable asthma cohorts while in the acute asthma cohort expression of CXCL1 and CXCL5 are up-regulated by 1.3 and 1.7-fold respectively when compared to healthy controls.

This suggests that *C. pneumoniae* infection of MoDCs from asthma patients induces increased expression of chemokines which attracts circulating DCs, neutrophils and eosinophils in the *C. pneumoniae* induced airway inflammation process.

Table 3.4: Differentially expressed Chemokines: healthy controls versus asthma cohorts. Using a criterion of Log Ratio ± 1 and p<0.05, significantly expressed chemokine genes were identified in the patient cohorts. "-" indicates not detected

Symbol	Entrez Gene Name	[a] Log Ratio (Healthy controls)	[a] Log Ratio (Acute asthma)	[a] Log Ratio (Stable asthma)	[b] Fold change	
					Acute asthma	Stable asthma
CCL1	chemokine (C-C motif) ligand 1	-	2.180	2.456	2.2	2.5
CCL3	chemokine (C-C motif) ligand 3	2.219	2.826	3.621	1.3	1.6
CCL3L1/CCL3L3	chemokine (C-C motif) ligand 3-like 1	3.662	4.628	5.953	1.3	1.6
CCL4	chemokine (C-C motif) ligand 4	-	-	1.573	-	1.6
CCL5	chemokine (C-C motif) ligand 5	2.318	3.331	4.833	1.4	2.1
CCL20	chemokine (C-C motif) ligand 20	4.303	4.447	6.708	1.0	1.6
CXCL1	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	3.542	4.478	5.521	1.3	1.6
CXCL2	chemokine (C-X-C motif) ligand 2	4.574	4.772	6.083	1.0	1.3
CXCL5	chemokine (C-X-C motif) ligand 5	2.231	3.783	4.284	1.7	1.9

[a] **Log Ratio values represent gene expression ratios (expression level in *C. pneumoniae* AR39-infected MoDCs divided by the expression level in uninfected MoDCs) on the $\log_2$ scale.**

[b] **Fold change values refers to Log Ratio values for both acute and stable asthma divided by Log Ratio values for healthy controls. Positive fold change values indicates higher gene expression level in stable and acute asthma when compared to healthy controls**

ii. Colony-stimulating factors (CSFs) gene expression profiles in healthy controls versus asthma cohorts:

The CSFs are 18–70 kDa glycoproteins that bind to receptor proteins on the surfaces of hemopoietic stem cells, thereby activating intracellular signaling pathways that can cause the cells to proliferate and differentiate into mature WBCs (White Blood Cells) (Hamilton, 2008). As shown in Table 3.5, three different CSF genes were identified which were significantly expressed using the threshold criterion.

From Table 3.5, expression of GM-CSF and G-CSF is up-regulated by 1.9 and 2.5-fold in the stable asthma cohort compared to the healthy controls while selective induction of M-CSF is seen in the stable asthma cohort while expression of M-CSF is absent in the acute asthma cohort. Meanwhile, expression of GM-CSF and G-CSF is up-regulated by 1.3-fold in the acute asthma cohort (Table 3.5) when compared to healthy controls.

This suggests that *C. pneumoniae* infection of MoDCs from asthma patients induces increased expression of CSFs which can further contribute to the airway inflammation process in asthma patients

Table 3.5: Differentially expressed colony stimulating factors (CSFs): healthy controls versus asthma cohorts. Using a criterion of Log Ratio ± 1 and p<0.05, significantly expressed chemokines, CSFs and cytokine genes were identified in the patient cohorts. "-" indicates not detected

Symbol	Entrez Gene Name	[a] Log Ratio (Healthy controls)	[a] Log Ratio (Acute asthma)	[a] Log Ratio (Stable asthma)	[b] Fold change	
					Acute asthma	Stable asthma
G-CSF	granulocyte colony stimulating factor	1.064	1.391	2.660	1.3	2.5
GM-CSF	granulocyte-macrophage colony stimulating factor	3.975	5.170	7.577	1.3	1.9
M-CSF	macrophage colony stimulating factor	-	-	1.966	-	1.97

[a] **Log Ratio values represent gene expression ratios (expression level in *C. pneumoniae* AR39-infected MoDCs divided by the expression level in uninfected MoDCs) on the $\log_2$ scale.**

[b] **Fold change values refers to Log Ratio values for both acute and stable asthma divided by Log Ratio values for healthy controls. Positive fold change values indicates higher gene expression level in stable and acute asthma when compared to healthy controls**

iii. Airway remodelling genes in the asthma cohorts

Apart from inflammation in the airways, disordered anatomy of the lungs in asthma patients is a characteristic symptom which is defined as airway remodelling. As shown in Table 3.6, expression of airway remodelling genes were both selectively and highly expressed in the asthma cohorts compared to the healthy controls.

From Table 3.6, the stable asthma patients showed increased selective expression of ADAMs (Adisintegrin and metalloprotease domain 8, 9 and 17), MMPs (Matrix metalloproteinase-3 and 19) and PAI-1 (Plasminogen activator inhibitor-1) by 2.2-5-fold.

Moreover, expression of other airway remodelling genes such as MMP-7, MMP-10, SerpinB2 (Serpin peptidase inhibitor member 2) and VEGF-A (Vascular endothelial growth factor A) were up-regulated in the stable asthma patients by 5-11-fold while these same genes were up-regulated in the acute asthma cohort by 2.9-8.5-fold.

This suggests that *C. pneumoniae* infection of MoDCs from asthma patients induces a selective or an increased expression of airway remodelling genes which can act to induce structural aberration in airways of asthma patients.

Table 3.6: Selectively expressed genes in healthy controls versus asthma cohorts. Using a criterion of 2-fold ± expression and p<0.05, selectively expressed genes were identified in the asthma patient cohorts compared to the healthy controls. Values represent mean fold changes in expression of the genes with negative numbers indicating down-regulation while positive numbers indicate up-regulation. "-" indicates not detected

Differentially and selectively expressed genes in normal vs asthma				
Gene Symbol	Gene name	Healthy controls	Acute asthma	Stable asthma
ADAM8	A disintegrin and metallopeptidase domain 8	-	-	3.3
ADAM9	A disintegrin and metallopeptidase domain 9	-	-	2.2
ADAM17	A disintegrin and metallopeptidase domain 17	-	-	2.7
MMP-1	Matrix metalloproteinase-1	-	4.4	10.3
MMP-3	Matrix metalloproteinase- 3	-	-	5
MMP-7	Matrix metalloproteinase-7	2.8	4.1	6.6
MMP-10	Matrix metalloproteinase-10	2.7	5.7	11
MMP-19	Matrix metalloproteinase-19	-	-	3
PAI-1	Plasminogen activator inhibitor-1	-	-	3.5
SERPINB2	Serpin peptidase inhibitor member 2	4.3	8.5	10.75
VEGF-A	Vascular endothelial growth factor A	-	2.9	5.22

iv. *Other immune response genes:*

Genes related to immune responses such as CCR7 (C-C chemokine receptor type 7), IDO (Indoleamine 2,3-dioxygenase-1) were selectively expressed in the asthma cohorts (Table 3.7).

The stable asthma cohort displayed selective expression of genes related to prostaglandin signaling pathway such as PGES (Prostaglandin E synthase), PGES2 (Prostaglandin E synthase 2) and PGE2R4 (Prostaglandin E receptors 4) which is consistent with findings from other studies (Fogel-Petrovic et al., 2004; Long et al., 2004) who have reported increased PGE2 synthesis in MoDCs from asthma patients in response to LPS stimuli.

Genes related to immune responses were also found to be selectively expressed in the healthy controls. As shown in table 3.7, genes related to antiviral defence such as OAS (2,5-oligoadenylate synthetase), PKR (Protein Kinase R) and MxA (myxovirus resistance protein 1) were expressed selectively in the healthy controls which were absent from the asthma cohorts. MxA, PKR and OAS are interferon stimulated genes secreted in response to IL-29 and IFN-α (Chai et al., 2011). Expression of IFN-α from microarray data was absent as did not reach statistical significance (Appendices, supplementary table 7) while expression of IL-29 was upregulated in all the patient cohorts (Appendices, supplementary table 7).

Table 3.7: Selectively expressed other immune response genes in healthy controls versus asthma cohorts. Using a criterion of 2-fold ± expression and p<0.05, selectively expressed genes were identified in the asthma patient cohorts compared to the healthy controls. Values represent mean fold changes in expression of the genes with negative numbers indicating down-regulation while positive numbers indicate up-regulation. "-" indicates not detected

Gene Symbol	Gene name	Healthy controls	Acute asthma	Stable asthma
CCR7	C-C chemokine receptor type 7	-	5.56	7.3
IDO	Indoleamine 2,3-dioxygenase-1	-	9.12	12.36
MxA	myxovirus resistance protein 1	6.56	-	-
OAS	2,5-oligoadenylate synthetase	3.68	-	-
PGE2R4	Prostaglandin E receptors 4	-	-	2.26
PGES	Prostaglandin E synthase	-	-	2.36
PGES2	Prostaglandin E synthase 2	-	-	-2.2
PKR	Protein kinase R	2.62	-	-

Chapter Four: Discussion

Chlamydia pneumoniae has been long associated with asthma, wheeze and asthma exacerbations in both adults and children (Black et al., 2000; Hahn et al., 1991; Hahn and McDonald, 1998; Johnston and Martin, 2005). Detection rates of this bacteria can be as high as 70% in patients with asthma (Hansbro et al., 2004), and serological positivity rates are often 40–60% (Kocabas et al., 2008), indicating a possible causative role of *C .pneumoniae* infection in asthma pathogenesis although none of the studies has described the mechanisms behind this *C. pneumoniae*-induced asthma. The respiratory pathogen *C. muridarum* has been studied in murine asthma models to test the hypothesis that upon infection it can induce allergy associated Th2 responses (Horvat et al., 2007; Horvat et al., 2010a; Horvat et al., 2010b; Kaiko et al., 2008). However, data from human studies are very limited with most studies having used cross-sectional methodology; there is little prospective data and evidence of association is largely based on sero-epidemiological studies.

DCs have been identified in dense networks throughout the epithelium of the respiratory tract, including the nose, nasopharynx, large conducting airways, bronchi, bronchioles, and alveolar interstitium which positions them as first responders to incoming respiratory pathogens (Gill, 2012). Respiratory pathogens often manipulate DCs to mount maladaptive immune responses which prevent optimal clearance of respiratory pathogens and contribute to the immunopathology in the lungs, as seen in asthma (Lambrecht, 2008). As MoDCs are active participants in host defence against infectious stimuli entering the airways, it was important to determine the extent of immunomodulatory effects of *C. pneumoniae* infection on MoDCs. In this thesis the comparative effect of *C. pneumoniae* infection on innate immune responses was explored using MoDCs isolated from healthy controls, acute asthma and stable asthma patients. This was done to deduce whether predisposition to asthma resulted in exaggerated inflammatory responses to *C. pneumoniae* infection. Although, there is increasing knowledge of the immunobiology of MoDCs, they are not well studied in the context of bacterial exacerbations of asthma. This study has shown that MoDCs from asthma patients respond to *C. pneumoniae* infection by significantly altering MoDC maturation (Figure 3.10) along with increasing cytokine secretion (Figure 3.11), chemokines (Table 3.4) and colony-stimulating factors (Table 3.5) gene expression compared to healthy controls. Moreover, *C. pneumoniae* infected MoDCs from asthma patients (both stable and acute) also show a selective expression of airway remodelling genes (Table 3.6) which can drive the structural aberrations in asthma patients.

C. pneumoniae can infect MoDCs *in vitro* with altered morphology and growth kinetics. In contrast to infected bronchial epithelial cells, *C. pneumoniae* infections in MoDCs do not form large contained inclusions (Figure 3.4) along with inability to support replication (Figure 3.3, panel A) and the formation of viable progeny (Figure 3.3, panel B). This finding is consistent with previous reported studies (Wittkop et al., 2006) which demonstrated the absence of *C. pneumoniae* growth in human MoDCs. This observation is in contrast in the murine *C. muridarum* infection of murine MoDCs that leads to the formation of viable inclusions and infectious progeny during infection (Kaiko et al., 2008; Rey-Ladino et al., 2007). To prevent completion of intracellular pathogen replication, innate immune cellular responses lead to apoptosis and cell death of infected cells. Previous studies have shown that *C. pneumoniae* survival in human PBMCs (Geng et al., 2000) and epithelial cells (Aso, 2007) is dependent on the inhibition of apoptosis by bacterial mediated mechanisms. However, this study shows that during infection MoDCs underwent significant apoptosis (Figure 3.6, panel A) and cell death (Figure 3.6, panel B), which is dependent on live but not killed organisms. Therefore MoDCs

may utilise a unique microbial response pathway that rapidly activates apoptosis to limit *C. pneumoniae* replication inside MoDCs and prevent dissemination outside the lung. This observation is supported by the suggestion that apoptosis may be promoted in human immune cells in an effort to limit immune response (Wolf, 2011). MoDCs, from the patient cohort, did undergo cell death in response to *C. pneumoniae* infection but they were constantly in the range of 50-60% (data not shown) with no differences in MoDC cell death observed between the groups in the patient cohort.

To deduce the mechanism behind this *C. pneumoniae* induced cell death in MoDCs, genome-wide transcriptional analysis was employed which identified up-regulation of inflammatory transcription factors AP-1, CREB-1 and NF-KB (Table 3.3) along with expression of other immune response genes such as cyclin-dependent kinase inhibitor 1 (p21) and Heat Shock protein 70 (HSP70) (Table 3.3). The cell cycle arrest gene p21 is induced in response to cellular stress and activated through activated through NF-KB -dependent pathway (Basile et al., 2003). Hence, expression of p21 through NF-KB -dependent pathway may regulate the observed effect of cell death in *C. pneumoniae*-infected MoDCs. Although NF-*κ*B is generally regarded as anti-apoptotic, as it protects cells from apoptosis by promoting expression of survival factors, such as members of the inhibitor of apoptosis (IAP) family (c-IAP1, c-IAP2, XIAP) and the Bcl-2 homologues, Bfl-1/A1 and Bcl-x_L (Ravi et al., 2001). It is now apparent that in particular contexts, and especially in response to cellular stress, NF-*κ*B acts to promote apoptosis (Fan et al., 2008). HSP70 release from MoDCs are known to be triggered under necrotic but not apoptotic cell death conditions (Basu et al., 2000) which can activate the NF-KB pathway (Ryan et al., 2000). Activation of NF-κB increases transcription of p53 which in turn induces transcriptional activation of target gene, p21 leading to subsequent cell cycle arrest and apoptosis. NF-*κ*B enhances p53 transcriptional function by p53 phosphorylation (Ryan et al., 2000) which in turn p21 expression and consequent cell cycle arrest and apoptosis (Basile et al., 2003). This suggest that apoptosis in *C. pneumoniae* infected MoDCs is an important host innate defence mechanism mediated by activation of NF-κB transcription factor which in turn induces transcriptional activation of target gene, p21 leading to subsequent cell cycle arrest and apoptosis. The subsequent cell cycle arrest by p21 prevents intracellular replication of *C. pneumoniae* in MoDCs. Induction of inflammatory transcription factors AP-1, CREB-1 and NF-KB (Table 3.3) also requires activation of pathogen recognition receptors (PRRs) such as TLR2 and TLR4 (Zhang and Ghosh, 2001), however, gene expression data in this study showed a downregulation of TLR2 and TLR4 along with NOD1 (Appendices, supplementary table 7) which in contrast with previous reported studies (Njau et al., 2009a; Zaharik et al., 2007). Further validation studies needs to be conducted on *C. pneumoniae*-infected MoDCs to deduce whether TLR2 or TLR4 pathways were activated. This can be done using TLR signaling assays as described by Burger-Kentischer and colleagues (Burger-Kentischer et al., 2010).

MoDCs are professional antigen presenting cells, as such play an important role in immune activation. A hallmark of human MoDC maturation is the up-regulation of co-stimulatory molecules including CD80, CD83 CD86 and HLA-DR that enable them to activate naive T cells through antigen presentation on HLA-DR. Several lines of evidence indicate that bacteria and viruses have evolved strategies to evade immune surveillance and in particular to block DC function (Khan et al., 2012). Intracellular bacterial pathogens like *Mycobacterium tuberculosis, Mycobacteria avium, M. leprae, Salmonella typhi (S. typhi), Helicobacter pylori (H. pylori)* have been shown to infect MoDCs and modulate expression of co-stimulatory molecules CD80 and CD86 (Khan et al., 2012).

In this study, MoDCs infected with live *C. pneumoniae* underwent a distinct maturation profile characterised by a selective down-regulation of maturation marker CD80 while leaving the expression of other maturation markers unaltered (Figure 3.5). Results revealed that only exposure to live *C. pneumoniae* induces selective down-regulation of CD80 while this effect is absent from MoDCs exposed to UV- and heat-killed *C. pneumoniae* (Figure 3.7). CD80 expression on MoDCs is preferentially associated with Th1 type responses (Hammad et al., 2001) while CD86 expression on MoDCs is known to skew T cell responses to Th2 phenotype (Hsu et al., 2002). Studies in murine models have shown that Th1 responses are effective in clearance of *C. muridarum* respiratory infection through elevated pulmonary IFN-γ production (Jupelli et al., 2008) while Th2 responses fail to clear chlamydial respiratory infections (Wang et al., 1999). This suggests that live *C. pneumoniae* selectively targets CD80 molecule for down-regulation in MoDCs to prevent induction of Th1 response and ensure its survival.

Previously published studies (Flego et al., 2012; Kis et al., 2008; Njau et al., 2009a; Njau et al., 2009b; Wittkop et al., 2008; Wolf, 2011) investigating interaction of human MoDCs with *C. pneumoniae* did not observe selective down-regulation of CD80. This discrepancy may be primarily dependent on the strain of *C. pneumoniae* used to infect MoDCs as differences in virulence of *C. pneumoniae* strains has been reported based on pathology induced in a murine model of infection (Sommer et al., 2009).

During infection, immune activation by MoDCs has shown to determine specific T cell phenotypes and immune response bias. The phenotypic modulation of MoDCs by allergens, including *Chlamydia*, plays a pivotal role in enhancement of Th2 responses in asthma (Hammad et al., 2001) as shown by murine studies (Kaiko et al., 2008). This study sought to examine the differences in phenotypic modulation of maturation markers on MoDCs, isolated from healthy, acute asthma and stable asthma cohorts, in response to *C. pneumoniae* infection. No differences in the numbers of recovered MoDCs, after culture with IL-4 and GM-CSF, were observed between the patient groups as expression of IL-4Rα on MoDCs from patient groups did not reveal any differences (Appendices, Supplementary table 7). Humans, both healthy and asthma patients are exposed to everyday environmental stimuli such as allergens, pollution and biological entities such as respiratory infections which combined together can elicit substantial immune changes in the lungs and may affect circulating blood monocyte populations through release of inflammatory mediators. This study observed significant changes in the basal expression levels of MoDC maturation markers CD80, CD83, CD86 and class II Human Leukocyte Antigen (HLA-DR) between patient cohorts, prior to infection with *C. pneumoniae*.

MoDCs generated from blood samples of the asthma cohorts displayed a significantly increased expression of maturation markers CD80, CD83 and CD86 compared to healthy controls (Figure 3.9). This increase in expression of DC maturation markers in the asthma cohort can partly be attributed to release of immune mediators released by bronchial epithelial cells from asthma patients which can act on circulating monocyte population in the bone marrow, to arm cytokine-driven differentiation of monocytes into MoDCs with anti-microbial defences that can be rapidly employed to encounter inhaled allergens or pathogens (Rate et al., 2012; Rate et al., 2009). The airway epithelium serves as a physical barrier between the external environment and the host and it possess numerous pattern recognition receptors (PRRs). Upon exposure to numerous environmental stimuli, such as allergens, infectious agents, pollutants, and oxidants, it can release inflammatory

mediators (Hammad and Lambrecht, 2008; Lambrecht and Hammad, 2010) which can act to recruit various immune cells and stimulate the bone marrow to induce production of immune cells (Fujii et al., 2002). Although stable asthma patients can control their symptoms by use of medications, studies have shown that bronchial biopsies from stable asthma patients contain increased baseline levels of eosinophilic inflammatory mediators, IL-8, IL-6 and inflammatory lipid mediators such as oxylipins (Lundstrom et al., 2012; Riise et al., 1996; Zietkowski et al., 2010). Hence, presence of inflammatory mediators in asthma airways might affect phenotypic commitment of monocytes to MoDCs with higher expression of multiple immune surveillance genes and DC maturation markers (Rate et al., 2012; Rate et al., 2009).

Although MoDCs generated from asthma patients displayed a significantly increased expression of CD80, CD83 and CD86, HLA-DR expression significantly differed between acute and stable asthma patients. HLA-DR expression was significantly high in MoDCs from the stable asthma patients compared to both healthy controls and acute asthma patients (Figure 3.9). In this study, blood samples from the acute asthma patients were collected during an onset of severe asthma exacerbation that could be triggered by multiple environmental factors. The causes of exacerbations were unknown at the time of the study as the patients did not undergo diagnostic procedures following treatment for asthma exacerbations. The state of asthma exacerbations could have potentially affected the circulating monocyte precursor population influencing their differentiation into MoDCs with altered characteristics (Sanchez-Torres et al., 2001; Shortman and Naik, 2007). Studies have shown that during an episode of ongoing systemic inflammation, a decreased expression of HLA-DR on monocytes is a hallmark of altered immune status in patients with a systemic inflammatory response as this acts to drive monocytes towards a protective scavenging phenotype with immunosuppresive function (Kim et al., 2010; Quimby et al., 2010; Yang et al., 2008). Although the lung is the main site of inflammation, there is substantial evidence that spillover of inflammatory mediators into the circulation is generally considered to be the source of this systemic inflammation (Wouters et al., 2009) causing activation of monocyte population during an episode of acute asthma exacerbation (Subrata et al., 2009a). Hence, the state of ongoing inflammation in acute asthma cohorts might explain the differences in HLA-DR expression when compared to other patient cohorts. Overall, the production of inflammatory mediators in airways from asthma patients might induce alterations in the biology of monocytes which effects differentiation of monocytes to MoDCs with higher expression of maturation markers CD80, CD83 and CD86 compared to healthy controls. However, HLA-DR expression is differentially modulated in MoDCs, generated from acute asthma and stable asthma patients, as presence of systemic inflammation in acute asthma patients activates circulating monocytes which switches them to low HLA-DR expression. These can have implications as it suggests that in presence of GM-CSF/IL-4-enriched cytokine milieu characteristic of the atopic asthmatic airway mucosa, monocytes can differentiate into MoDCs and enhance adaptive immune responses.

During infection with *C. pneumoniae*, human MoDCs undergo changes in the expression of maturation markers, and this is dependent on healthy or asthma disease status. *C. pneumoniae* infection induced a threefold down-regulation of surface marker CD80 in both the healthy controls and the asthma patients (Figure 3.10). In contrast, microarray profiling (Table 3.1) indicates an up-regulation of CD80 marker in response to *C. pneumoniae* infection in both the healthy controls and the asthma patients. This disprecancy in CD80 expression at RNA and protein level may be mediated by *Chlamydia* proteases such as CPAF (Chlamydial Protease-like Activity Factor), tail-specific protease

(Tsp), and chlamydial high temperature requirement protein A (cHtrA) (Zhong, 2011) which are known to degrade host cell proteins (Fan et al., 2002). In contrast, a significant up-regulation of DC maturation marker CD83 was observed in the stable asthma cohort in both flow cytometry and microarray data. CD83 is stored in preformed intracellular vesicles in MoDCs for rapid expression upon activation and provide potent co-stimulatory signals to naive and memory T-cells (Aerts-Toegaert et al., 2007; Cao et al., 2005; Hirano et al., 2006). Hence, a combined down-regulation of CD80 and up-regulation of CD83 is observed on *C. pneumoniae* infected MoDCs from stable asthma patients and whether this leads to a potential Th2 stimulatory property has to be further verified using Mixed Lymphocyte Reaction (MLR) assays in the future.

MoDC maturation leads to the production of cytokines and chemokines in turn mediating immune responses. Cytokines play a key role in orchestrating the chronic inflammation of asthma by recruiting, activating, and promoting the survival of multiple inflammatory cells in the respiratory tract (Barnes, 2008). It is not simple to classify the numerous cytokines that are potentially involved in asthma because of their pleiotropic nature and overlapping properties. However, with regard to the specific abnormalities of asthma and to our current understanding of the pathogenesis of asthma, they may be grouped as follows: Lymphokines (IL-2, IL-3, IL-4, IL-5, IL-13, IL-15, IL-16, IL-17), pro-inflammatory cytokines (IL-1β, TNF-α, IL-6, G-CSF, GM-CSF, M-SCF), anti-inflammatory cytokines (IL-10, TGF-β), chemokines (CCL5, CCL2, CCL8, CCL7, CCL13, CCL12, CCL3, CCL11, IL-8) and growth factors (PDGF, FGF, EGF, IGF) (Renauld, 2001).

Most studies have focused on the effects of Th2 cytokines IL-4, IL-5, IL-9 and IL-13 due to their role in driving asthma, a handful of studies has also investigated the effects of TNF-α, IL-1β, IL-6 and IL-8 in human asthma. In this project, MoDC-derived cytokines were investigated which can trigger clinical symptoms of asthma. Results (Figure 3.11) demonstrated that the asthma cohorts displayed a significant increase in secretion of TNF-α and IL-1β. Both IL-1β and TNF-α have been demonstrated to act directly on airway smooth muscle cells and perpetuate asthmatic airway inflammation by altering airway smooth muscle hyper responsiveness to contractile agonists (Amrani et al., 1997; Shore et al., 1997). This effect is mediated through enhanced elevation of intracellular calcium in airway smooth muscle cells that cause an increased contractile response. Thereby, secretion of TNF-α and IL-1β derived from *C. pneumoniae*-infected MoDCs in asthma can influence airway smooth muscle contraction responses when present in the vicinity of airways in asthma.

Results from this study also demonstrated that a significant production of cytokines IL-4, IL-10, IL-6, and IL-8 were detected from *C. pneumoniae* infected MoDCs derived from the asthma cohort (stable and acute (Figure 3.11). However, when comparing the stable and acute asthma patients this study found that *in vitro* cytokine secretion profiles in the acute asthma patients were low when compared to stable asthma patients. This possibly could be due to differences in half-life of cytokine mRNA versus the protein. Mammalian cytokine mRNA contains an AU rich element (ARE) in the 3' noncoding region which promotes the rapid degradation of mammalian cytokine mRNAs while cytokine proteins undergo ubiquitin mediated degradation (Laroia et al., 2002). Cytokines are important regulators of immunity and the development of asthmatic disease characterised by a Th2 phenotype. The observed increase in both IL-4 and IL-10 (Figure 3.11) while absence of IL-12 secretion are important for generation of Th2 cells (Demedts et al., 2005; Vandenbroucke et al., 2011). Moreover IL-6 secretion which is significantly increased in the asthma cohorts, interferes

with IFN-γ production by CD4 T cells through up-regulation of SOCSs (SOCS1 or SOCS3) that provide a negative feedback and diminish IFN-γ signalling thus inhibiting Th1 development (Diehl et al., 2000). IL-8 is a powerful chemoattractant and activator of neutrophils and recent evidence shows IL-8 also mediates eosinophils migration, however the role of IL-8 in eosinphillic asthma is unclear (Ma et al., 2009). It is produced by a wide range of cell types like monocytes, macrophages, MoDCs, fibroblasts and keratinocytes. Two main forms of asthma dependent on cellular infiltration are recognised. Eosinophilic asthma is driven by presence of IL-5 is derived from Th2 cells and forms part of the adaptive immune system, whereas non-eosinophilic (or neutrophilic asthma) observes an increase in neutrophil cell counts correlating with IL-8 levels in the sputum samples of asthma patients (Simon, 2001; Simon et al., 1997; Simpson et al., 2007; Simpson et al., 2006). This data is evidence that *C. pneumoniae* infection of MoDCs leads to the production of pro-inflammatory cytokines (IL-1β, IL-4, IL-6, IL-8, IL-10 and TNF-α) and may contribute to the pathogenesis of asthma through driving a Th2 phenotype development, neutrophilic recruitment and bronchial hyper-reactivity responses (Figure 4.1).

Although expression of IL-12 was undetectable in supernatants derived from *C. pneumoniae*-infected MoDCs from our patient cohort (Figure 3.11), it could be possible that a preferential production of IL-23 was induced instead of IL-12. IL-12 is a heterodimeric cytokine composed of two disulfide-linked subunits, IL-12p35 and IL-12p40 encoded on different chromosomes while IL-23, another heterodimeric cytokine, is composed by the specific p19 subunit (IL-23A) and the IL-12p40 chain that is shared with IL-12 (Watford et al., 2004). Despite a structural similarity between IL-12 and IL-23, they perform opposing roles as IL-12 promotes the differentiation of naive CD4 T cells into Th1 cells (Romagnani, 1999) while IL-23 regulate the establishment and clonal expansion of Th17 cells (Stritesky et al., 2008). From table 3.2, expression of IL-23A gene was shown to be expressed in all the patient cohorts while expression of IL-12p40 gene was only expressed in the stable asthma cohort suggesting a possible production of IL-23 in stable asthma cohort. In murine models of asthma, overexpression of IL-23 induces Th17 cell-mediated neutrophilic airway inflammation along with upregulation of Th2 cell-mediated eosinophilic inflammation (Wakashin et al., 2008). However, Th17 cells are also known to be phenotypically unstable as recent studies have shown that they can trans-differentiate into Th1 cells (Annunziato et al., 2007) under high IL-12 levels (Nistala et al., 2010). This can have implication in host defence against *C. pneumoniae* infection as murine studies have shown that Th17 cells can promote Th1 cell immunity against pulmonary chlamydial infection through modulating DC function (Bai et al., 2009). Hence, future studies aimed at detection of IL-23 needs to be carried out on *C. pneumoniae*-infected MoDCs using cytokine ELISAs to deduce whether IL-23 induced Th17 cell generation promotes Th1 immunity against *C. pneumoniae* or susceptibility to *C. pneumoniae*-induced asthma.

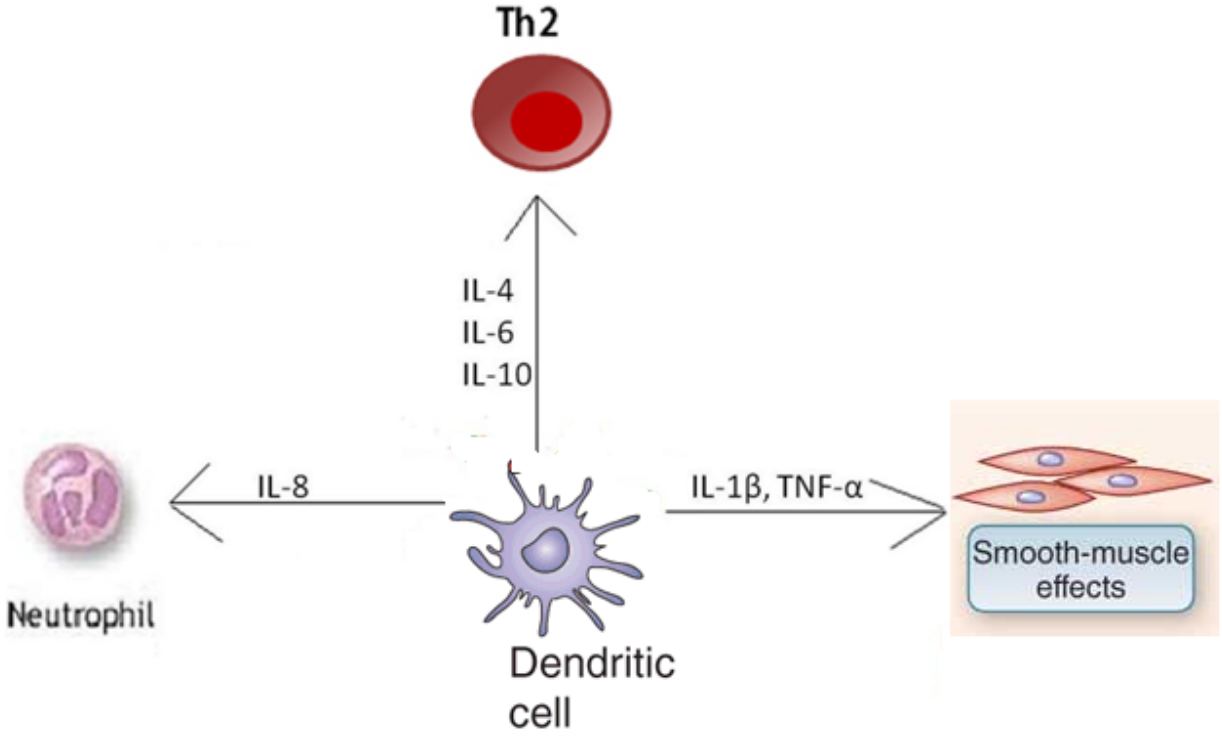

Figure 4.1 shows the effect of DC-derived cytokines on asthma. IL-4, IL-6 and IL-10 secretion by *C. pneumoniae* infected MoDCs can promote Th2 cell response while IL-8 secretion can act as a chemoattractant for neutrophils driving neutrophilic inflammation. Secretion of both IL-1β and TNF-α by *C. pneumoniae* infected MoDCs can promote contraction of airway smooth muscle cells. Figure modified from(Wenzel, 2012).

In addition to cytokines, a common pathological feature in asthma is the presence of a characteristic allergic airway inflammatory response involving extensive leukocyte infiltrate driven by the action of chemokines. Chemokines were significantly up-regulated in asthma cohorts when compared to healthy controls using microarray profiling. Chemokines are a family of small cytokines that signal through G-protein–coupled receptors and are classically associated with immune cell recruitment to sites of injury. They have been divided into four groups based on their molecular structure and the position of the first two cysteine residues: the CC and CXC (X=amino acid) and the less described C and CX3C families. At present, 16 CXC ligands (CXCL1–16) and 28 CC ligands (CCL1–28) have been identified (Lukacs, 2001). Migration of DCs from sites of inflammation to lymph nodes to initiate primary immune responses are mediated by CCR7 receptor under the influence of the CCR7 ligands CCL19 and CCL21 (Jakubzick et al., 2006). Similarly DCs are replenished in the lungs during ongoing pulmonary inflammation through the action of chemokines. CCL20 (MIP3α) expression was comparatively increased in the stable asthma cohort compared to the healthy controls. CCL20 acts as a DC chemoattractant that mediates the accumulation of immature DCs within the lungs in turn mediating immunity locally (Osterholzer et al., 2005).

Supporting a role for *C. pneumoniae* infection in driving both eosinophilic and neutrophilic asthma phenotypes, chemokines that specifically recruit each cell type was up regulated in the asthma cohorts compared to health controls during infection.

Eosinophil attracting chemokine genes, CCL1, CCL3, CCL4 and CCL5, were shown to be up-regulated in the stable and acute asthma patients by 1 to 2-fold when compared to healthy controls (Table

3.4). Both CCL3 (also known as Macrophage Inflammatory protein-1 alpha (MIP-1α)) and CCL5 (also known as RANTES) play an active role in recruiting eosinophils to the site of airway inflammation. Using murine asthma model, CCL3 and CCL5 were shown to be major eosinophil chemotactic factors produced during allergic airway responses and a significant reduction in pulmonary eosinophil chemotaxis was observed when CCL3 and CCL5 were neutralised *in vivo* using antibodies (Lukacs et al., 1996). Apart from chemotaxis, chemokines also mediate a range of proinflammatory effects on eosinophils including activation, and degranulation. Studies from murine models have shown that CCL1 and CCL4 can both act on eosinophils by binding to receptors CCR5 and CCR8 which can trigger release of inflammatory mediators from eosinophils (Oliveira et al., 2002). Similarly, neutrophil attracting chemokine genes, CXCL1, CXCL2 and CXCL5 genes were increased between 1.3-2-fold in the asthma cohorts when compared to healthy controls. These chemokines plays a crucial role in recruiting neutrophils in response to microbial infection and tissue injury as CXCL1, CXCL2 and CXCL5 together with IL-8 are defined as neutrophil attracting chemokines based on their presence in bronchial biopsies of COPD patients with an increased neutrophilia (Qiu et al., 2003). CXCL5 (C-X-C motif chemokine 5) promotes the influx of neutrophils and plays a major role in driving inflammation that exacerbates an existing asthmatic pulmonary environment (Kallal and Lukacs, 2008) while CXCL1 and CXCL2 are known to contribute to neutrophil trafficking to site of injury during inflammation (De Filippo et al., 2013). Thus, it appears that production of IL8 and chemokines by *C. pneumoniae*-infected MoDCs from asthma patients can promote the influx of neutrophils. This can play a major role in driving inflammation that exacerbates an existing asthmatic pulmonary environment (Newcomb et al., 2007). Collectively, these results (Table 3.4) suggests that *C. pneumoniae* infection of MoDCs from asthma patients induces increased expression of chemokines which attracts circulating DCs, neutrophils and eosinophils in the airway inflammation process (Figure 4.2).

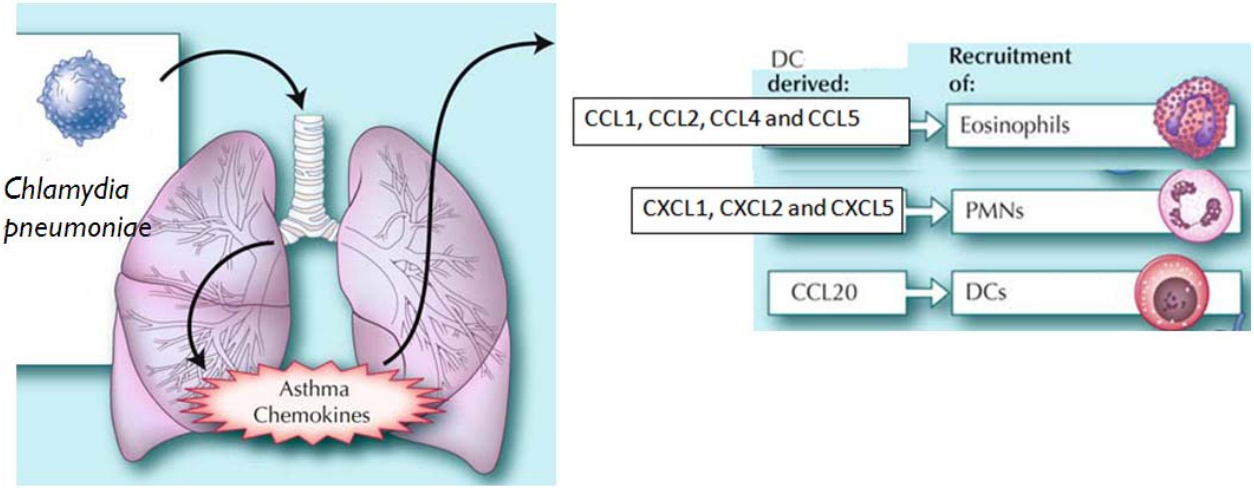

Figure 4.2: Chemokine genes up-regulated during *C. pneumoniae*-induced asthma exacerbation. *C. pneumoniae* infected MoDCs from asthma patients up-regulates the expressions of chemokine genes attracting eosinophils, neutrophils and immature MoDCs which subsequently contribute to the severity of the exacerbated asthmatic response. Figure adapted from (Kallal and Lukacs, 2008).

Growth factors such as colony stimulating factors (CSFs) are important regulators mediating haematopoietic stem cells proliferation and differentiation (Hamilton, 2008) and also immune cell survival (Molloy et al., 1995).

Expression of Granulocyte Macrophage Colony-stimulating Factor (GM-CSF), Granulocyte Colony-stimulating Factor (G-CSF) and Macrophage Macrophage colony-stimulating factor (M-CSF) genes were up regulated in the asthma cohorts compared to the healthy controls (Table 3.5). GM-CSF acts on haematopoietic progenitors to promote the production, maturation and release of eosinophils from the bone marrow as well as activating the eosinophils in the lung. Apart from production of immune cells from bone marrow, GM-CSF also prolongs eosinophil and neutrophil survival in the lung, by suppression of apoptosis (Simon, 2001). Increased presence of GM-CSF has been detected in bronchial biopsies from asthma patients (Sousa et al., 1993) while increased presence of G-CSF has been detected in serum samples from severe persistent allergic asthma patients (Yalcin et al., 2012). GM-CSF is produced by many immune cells including MoDCs and bronchial epithelial cells can also produce GM-CSF in response to allergen challenge (Ritz et al., 2002). In mouse models of lung inflammation, a neutralizing GM-CSF specific antibody ameliorates the asthma symptoms by reducing airway hyper-responsiveness, while bronchial eosinophilia is decreased in GM-CSF deficient mice (Dijkstra et al., 2009). In contrast, G-CSF stimulates the growth of neutrophils in the bone marrow and can influence the function of mature neutrophils by enhancing superoxide production, phagocytosis, and bacteriocidal killing (Roberts, 2005). Interestingly an increase in M-CSF expression was seen in the stable asthma but not the acute asthma patients. M-CSF is produced by endothelial cells, fibroblasts, and mononuclear phagocytes and regulates the survival, proliferation and is responsible for the survival, proliferation, differentiation and activation of macrophages at various stages of their development (Praloran, 1991). Activation of monocytes/macrophages by M-CSF an important part in asthma because it can potentially promote macrophage infiltration while G-CSF can promote neutrophil infiltration in airway inflammation process (Figure 4.3).

To summarise, increased expression of genes encoding for CSFs in *C. pneumoniae*-infected MoDCs from asthma patients can exacerbate airway inflammation in asthma by ensuring survival of activated macrophages, eosinophils and neutrophils that allows for increased production of inflammatory mediators in the airways (Figure 4.3).

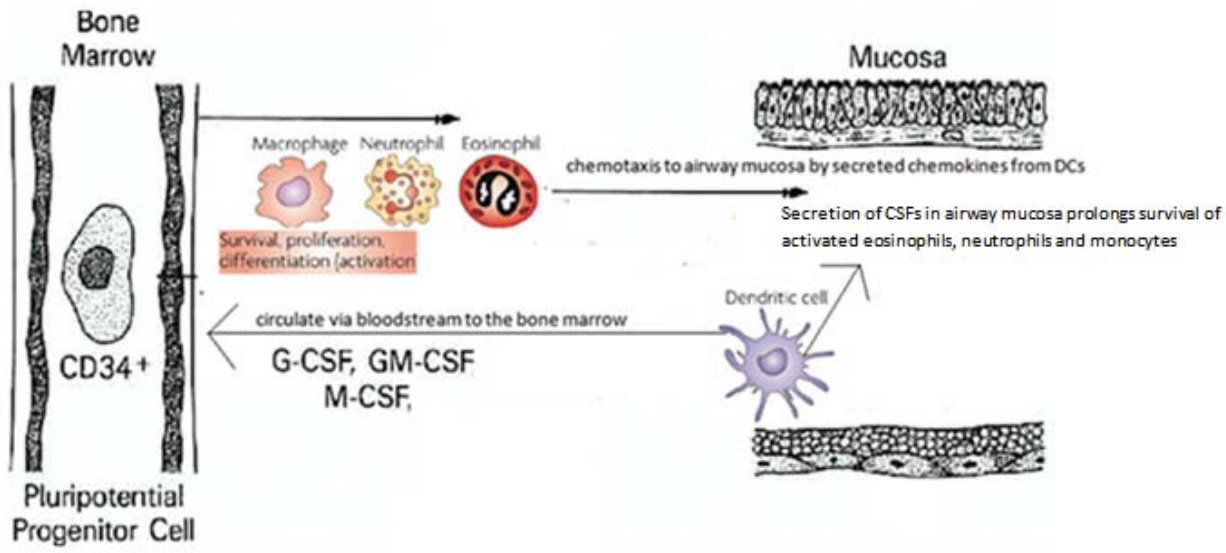

Figure 4.3: Action of CSFs on airway inflammation in *C. pneumoniae*-induced asthma exacerbation. Secreted GM-CSF, G-CSF and M-CSF from *C. pneumoniae*-infected MoDCs from asthma patients can act locally in the airways by prolonging survival of activated monocytes, eosinophils and neutrophils which contributes to ongoing airway inflammation process. Secreted CSFs from MoDCs can also circulate from airways to bone marrow, via bloodstream circulation, where it can stimulate differentiation of eosinophils, neutrophils and macrophages from haematopoietic progenitor cells. Figure adapted from (Hamilton, 2008).

Induction of immune tolerance is critical, during both physiologic conditions and inflammatory conditions such as asthma, to maintain the protective role of DCs. Expression of Indoleamine 2,3-dioxygenase-1 (IDO) and C-C chemokine receptor type 7 (CCR7) was selectively up-regulated in the asthma cohorts (Table 3.7). IDO degrades the indole moiety of tryptophan which greatly affects T-cell proliferation and survival (Grohmann et al., 2003) . This acts to suppress T-cell responses and promote tolerance (Mellor and Munn, 2004). IDO is also previously been shown to be expressed in response to *C. pneumoniae* infection in MoDCs (Njau et al., 2009a) as it acts to restrict *C. pneumoniae* growth in MoDCs. IDO-expressing DCs are shown to correlate with inhibition of experimental asthma in murine models (Hayashi et al., 2004) as intratracheal instillation of IDO-expressing murine DCs led to reduction of allergic airway inflammation. The mechanism behind this abrogation was reduction in total cell counts in BALF and IDO-expressing DCs induced Th2 cell apoptosis and reduced Th2 cell activation and allergic airway inflammation in OVA sensitized mice. CCR7 is a receptor for ligands CCL19 and CCL21 and its expression is associated with directed migration of MoDCs to lymph nodes (Jakubzick et al., 2006) where the antigen-loaded DCs present to naive T-cells and induce T-cell proliferation. Hence, CCR7 dependent migration of DCs, expressing IDO, to lymph nodes can possibly reduce airway inflammation in asthma through inhibition of T-cell proliferation along with controlling *C. pneumoniae* infection in DCs.

Interferons (IFNs) belong to the large class of glycoproteins known as cytokines that exhibit potent antiviral and immunoregulatory activities, and are classified into three major groups: type I, II and III (Pestka et al., 2004). They act on immune cells to induce production of various IFN stimulated genes (ISGs) (Sen and Sarkar, 2007) such as MxA, OAS and PKR which has many antiviral properties. Expression of MxA, PKR and OAS genes have been shown to be selectively upregulated by 2.6-6.5 fold in the healthy controls but absent from the asthma cohorts (Table 3.7). MxA, OAS and PKR

expression are known to be induced by interferons, IFN-α and IL-29 (Chai et al., 2011) however detection of IL-29 and IFN-α from our gene expression data (Appendices, supplementary table 7) did not meet the threshold criterion as described in the Results section. Absence in expression of MxA, PKR and OAS genes in the asthma cohorts can partly be attributed to impaired inability of immune cells from asthma patients to interferons. Studies have shown that PBMCs from asthma patients have an impaired ability to release IFNs in response to viral infection (Forbes et al., 2012; Gehlhar et al., 2006) which might explain the lack of expression of these antiviral protein genes in the asthma patients (Table 3.7).

Airway remodelling is defined as structural changes occurring in the airways that can lead to partially reversible airflow obstruction and accelerated lung function decline, as seen in asthma. The loss of surface epithelial integrity leading to damage to the airway epithelium is driven by Matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinases (ADAMs) secreted from surrounding immune and structural cells. These include ADAM8, ADAM9, ADAM17, MMP-1, MMP-3, MMP-7, MMP-10, MMP-19, PAI-1, SerpinB2 and VEGF-A (Table 3.6). This study identified an up regulation of airway remodelling genes in the asthma cohort.

The ADAMs are a family of transmembrane and secreted proteins with important roles in regulating cell phenotype via their effects on cell adhesion, migration, proteolysis and signalling. Though all ADAMs contain metalloproteinase domains, in humans only 13 of the 21 genes in the family encode functional proteases (Edwards et al., 2008). ADAM 8, 9 and 17 have proteolytic activity. ADAM8 is strongly induced by allergens and Th2 cytokines in the lung in experimental asthma (Foley et al., 2007). Surprisingly little is known about the contributions of ADAM proteinases to pathologies occurring in the airways of asthmatic patients. ADAM33 was the first member of the ADAM family to be linked to asthma (Knolle and Owen, 2009) but their role is still unclear. ADAM17 also known as tumor necrosis factor-α converting enzyme (TACE), is a membrane-bound enzyme that cleaves cell surface proteins, such as cytokines (e.g. TNFα), cytokine receptors (e.g. IL-6R and TNF-R), ligands of ErbB (e.g. TGFα and amphiregulin) and adhesion proteins (e.g. L-selectin and ICAM-1) (Scheller et al., 2011).

MMPs are zinc-dependent endopeptidases capable of degrading extracellular matrix molecules. Several subclasses of MMPs have been identified based on their substrate specificity; collagenases, gelatinases, stromelysins and membrane-type MMPs (Gueders et al., 2006). Inflammatory cells including macrophages, MoDCs, neutrophils, eosinophils, T cells and resident cells such as fibroblasts, epithelial cells and airway smooth muscle cells in the airways are capable of synthesising and releasing MMPs. The majority of MMPs are not expressed in normal healthy tissues but are expressed in diseased tissues that are inflamed or undergoing repair and remodelling. MMP-9 was the first MMP to be implicated in the pathology of asthma with increased levels detected in bronchoalveolar lavage fluid (Mautino et al., 1997), sputum induced by the inhalation of hypertonic saline (Cataldo et al., 2000) and in serum (Bosse et al., 1999) of asthmatic patients. In healthy lung MMPs and their physiological inhibitors, tissue inhibitors of matrix metalloproteinases (TIMPs), are produced in the respiratory tract by a panel of different structural cells. These activities are mandatory for many physiological processes including development, wound healing and cell trafficking. Inappropriate secretion of various MMPs by stimulated structural or inflammatory cells is thought to contribute to numerous lung diseases including asthma, chronic obstructive pulmonary

disease (COPD). Due to the proteolytic nature of MMPs in airway epithelium and the essential role they play in extracellular matrix turnover, selective MMP inhibitors can be tried for therapeutic aims in *C. pneumoniae* induced asthma. However, inhibitors targeting MMPs are still in clinical trials phase which makes it currently inaccessible to asthma patients (Hu et al., 2007). Doxycycline, an antibiotic used to treat chlamydial genital tract infections, has been shown to inhibit MMP-9 activity (Kaito et al., 2003). However, use of doxycycline in treatment for cystic fibrosis patients is still currently being addressed in clinical trials (Beringer et al., 2012).

Microvascular alterations in bronchial vessels and oedema are another feature of asthma which are characterised by growth and proliferation of new blood vessels and plasma leakage (Zanini et al., 2010b). These combined together can lead to thickening of the airways causing bronchoconstriction. Microvascular changes are mediated by release of growth factors in the airways by various invading inflammatory cells in the airways. Vascular endothelial growth factor (VEGF also referred to as VEGF-A), is a key regulator of blood vessel growth in the airways of asthma patients by promoting proliferation and differentiation of endothelial cells and inducing vascular leakage and increased permeability. Thus, VEGF has clinically relevant actions on the microvasculature in the airway of asthmatic subjects (Lee et al., 2011). The main source of VEGF in the airways are alveolar epithelial cells, bronchial epithelial cells, smooth muscle cells, fibroblasts and alveolar macrophages (Meyer and Akdis, 2013). Increased levels of VEGF has been identified from induced sputums of asthma patients (Asai et al., 2003) whilst bronchial biopsy specimens from asthma patients has identified increased presence of VEGF+ cells (Chetta et al., 2005) compared to healthy controls.

The role of VEGF in inducing asthma has been studied using a murine transgenic model where increased expression of VEGF was induced under the influence of the CC10 promoter (Lee et al., 2004). Increased expression of VEGF in the lungs of the transgenic mice led to development of asthma-like alterations and enhanced Th2 inflammation along with increased number of activated MoDCs in the lungs. Moreover, using an OVA-induced mouse asthma model, administration of ovalbumin was shown to increase DCs in the pulmonary tissues along with increased production of VEGF by Th2 cells and airway epithelial cells. This suggests that VEGF produced during innate immune responses can generate asthma-like inflammation and physiologic dysregulation of airways (Lee et al., 2004). Thereby, expression of VEGF from *C. pneumoniae*-infected MoDCs from asthma patients can further drive airway remodelling process by inducing microvascular changes (Figure 4.4). A potential use of VEGF inhibition for asthma therapy has been demonstrated in animal experiments however there have been no trials in humans investigating the therapeutic potential of VEGF in asthma (Meyer and Akdis, 2013).

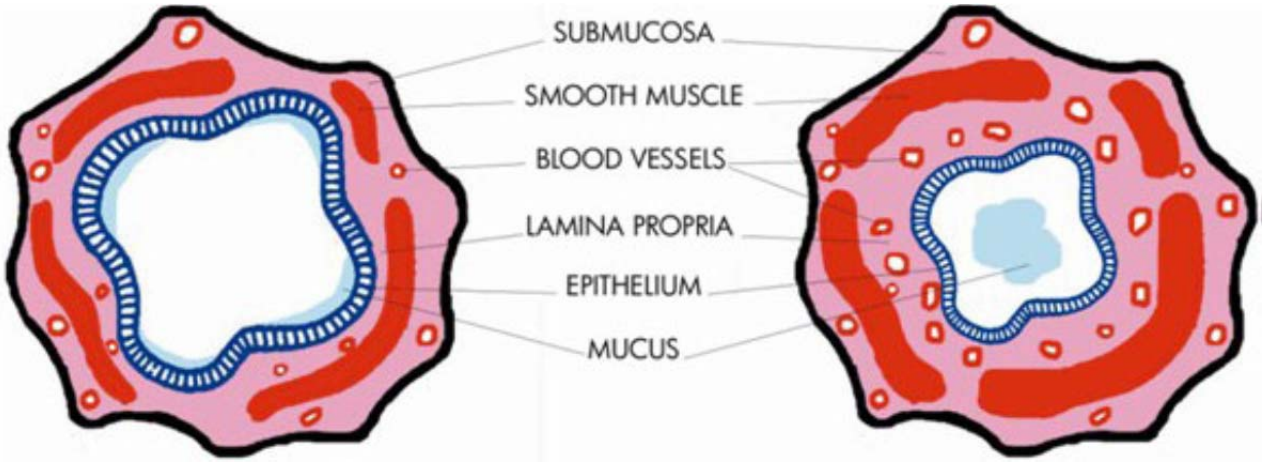

Fig. 4.4: Microvascular alteration by VEGF in asthma. Schematic picture of normal (left) and asthmatic airway (right), indicating the remodelling of compartments caused microvascular alterations induced by VEGF. Figure taken from (Zanini et al., 2010a)

Recent evidence indicates that chronic asthma may lead to tissue remodelling such as sub-epithelial fibrosis and extracellular matrix (ECM) deposition in the airways. Sub-epithelial fibrosis in asthmatic airways due to fibrin deposition leads to further thickening and this process is mediated by plasminogen activator inhibitors (PAIs) which form an important component of the coagulation system that down regulates fibrinolysis in the circulation (Mehta and Shapiro, 2008). PAI-1 is the main inhibitor of the fibrinolytic system and is known to play an essential role in tissue remodelling. Selective expression of SerpinB2 (also known as PAI-2) and PAI-1 was displayed by *C. pneumoniae*-infected MoDCs from the asthma cohort. SerpinB2 and PAI-1 are both members of the serpin (serine protease inhibitor) superfamily with an ability to inhibit tissue-type plasminogen activators (tPA) and urokinase-type plasminogen activators (uPA), prevents activation of plasmin, and promotes fibrin formation (Law et al., 2006). SerpinB2 and PAI-1 has been shown to be up-regulated from airway epithelial cells in asthma patients (Woodruff et al., 2007) which further strengthens its role in airway remodelling in asthma (Figure 4.5).

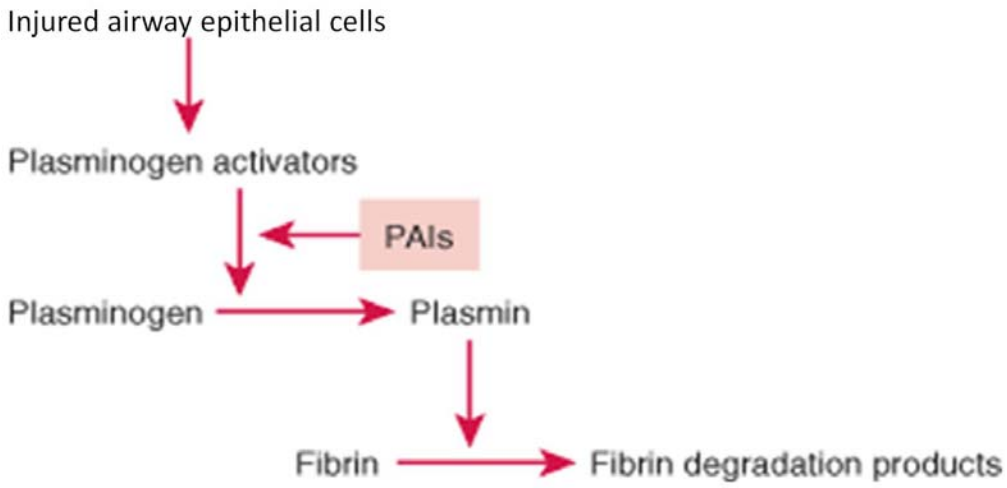

Fig.4.5: Fibrin deposition by PAIs. Fibrin deposition and fibrinolysis must be balanced during repair of injured airway epithelial cells. Injured airway epithelial cells release plasminogen activators (tissue plasminogen activator, urokinase), activating fibrinolysis. Plasminogen activators cleave plasminogen into plasmin, which dissolves clots. Fibrinolysis is controlled by plasminogen activator inhibitors (PAIs; eg, PAI-1). Figure modified from (Licona-Limon et al., 2013)

The findings of this study show that a significant up-regulation of cytokines, chemokines and growth factors along with selective expression of airway remodelling genes is observed in both stable and acute asthma patients during *C. pneumoniae* infection. Targeting these selective pathways might lead to better clinical management or new therapeutic treatments for *C. pneumoniae* induced asthma exacerbations. Currently, antibiotics targeting *C. pneumoniae* offer the most promising avenue for treatment of *C. pneumoniae* induced asthma exacerbations. Macrolides such as azithromycin and doxycycline are currently the treatment of choice in infections caused by *C. pneumoniae* (Hammerschlag and Kohlhoff, 2012; Xepapadaki et al., 2008) and use of macrolides has been shown to alter the development of clinical course of asthma (Friedlander and Albert, 2010; Hernando-Sastre, 2010; Xepapadaki et al., 2008; Zarogoulidis et al., 2011). Moreover, macrolide antibiotics can also modulate immune responses by acting on human MoDCs (Polancec et al., 2012) and PBMCs (Spits and Cupedo, 2012) as they reduce generation of Th2 inflammatory properties. Although use of antibiotics can eliminate *C. pneumoniae*, the bacterium is known to survive inside human lymphocytes (Yamaguchi et al., 2003) and CD14 monocytes (Gieffers et al., 2001) which were detected by presence of chlamydial DNA transcripts. This is possibly due to differences in pharmacokinetics of macrolides entering immune cells are different from macrolides entering *C. pneumoniae*-infected epithelial cells (Yamaguchi et al., 2003). Hence use of macrolides in treating *C. pneumoniae* induced asthma exacerbations needs to validated using both efficacy and safety data from larger patient numbers.

Thereby in an attempt to find novel therapeutic interventions that selectively targets *C. pneumoniae* infected DCs or immune cells without putting the patients at risk for asthma attack, it is important to focus on vaccine approaches for *C. pneumoniae*. A vaccine against *C. pneumoniae* would therefore not only reduce the incidence of the severe pathological complications of *C. pneumoniae* infection but also, and equally important, need to avoid generating persistence. This is particularly important considering the link between *C. pneumoniae* and atherosclerosis (Chatzidimitriou et al., 2012).

Limitations

The asthma patients recruited into the study also had been on asthma medications and the underlying effect of external factors such intake of steroid medications in the asthma cohort cannot be excluded as steroids are the mainstay of current asthma therapy (Edwards et al., 2006; Hansbro et al., 2008). Based on the patients history of asthma medications, patients were reported to be on interval medication such as an inhaled steroid and a sympathomimetic β2-agonist aerosol (bronchodilator - Ventolin, Bricanyl). Also use of a muscarinic receptor antagonist (Ipratropium, short-acting, or Tiotropium, long-acting), and preventive inhalation such as Cromones (Sodium Cromoglycate or Nedocromil) were reported in some of the patients. None of the stable asthma patients had a medication history of Omalizumab (IgE blocking antibody) or the leucotriene inhibitors. These asthma medications can exert various potent suppressive effects on MoDCs as listed in Table 4.1. In this study, the yield of recovered MoDCs between asthma patients and healthy controls did not vary as expression of IL-4Rα (IL-4 receptor α) was not detected from the gene array expression data (Appendices, supplementary Table 7). Nevertheless, the presence of these medications in the circulating monocyte population in the asthma patients can potentially exert various immunosuppressive effects influencing their immune responses to *C. pneumoniae*.

The acute asthma patients recruited into this study had an onset of severe asthma exacerbation, as described in the methods in Chapter 2. The cause of asthma exacerbations was not diagnosed during the time of the study and this can include various causes including viral infections. Studies have shown the presence of viral infections can induce various innate antiviral and atopic immunoinflammatory pathways in human PBMCs which act to precipitate and sustain asthma exacerbations (Subrata et al., 2009b). Hence the presence of any possible viral-induced asthma exacerbations in the acute asthma cohort could potentially influence some of the findings in this study.

As shown in Fig.3.15, unique genes were identified in the asthma cohorts which includes identifying 65 unique genes in the acute asthma cohort and 1615 unique genes in the stable asthma cohort. However majority of these genes were mainly related to downstream signaling molecules leading to cytokine production and TLR signaling pathways. Apart from immune signaling molecules, majority of the “unique” genes were also related to host cell cytoskeleton which is not not surprising as studies have shown that chlamydial infection of host cells are known to affect the host-cell cytoskeletal architecture (Campbell et al., 1989) and require actin polymerization for release of *Chlamydia* EBs by extrusion (Hybiske and Stephens, 2007).

Table 4.1:Asthma medications mode of action and their effect on immune cells or MoDCs.

Medications	Mode of Action	Effect on immune cells or DCs
short- and long-acting β2-adrenoceptor agonists (SABAs and LABAs)	Bind to Beta-adrenergic receptors coupled to G protein which activates cAMP and causes a change in intracellular calcium concentration which opens potassium channels causing airway smooth muscle relaxation (Panina-Bordignon et al., 1997)	Intranasal inoculation of murine DCs treated with hort- and long-acting b2-agonists do not modify DCs-induced allergic airway inflammation (Panina-Bordignon et al., 1997)
Theophylline	acts both a cAMP phosphodiesterase (PDE) inhibitor and an adenosine-receptor antagonist which prevents bronchoconstriction (Ito et al., 2002)	Inhibits monocyte differentiation into MoDCs by apoptosis. Induces HDAC activity to reduce inflammatory gene expression (Yasui et al., 2009).
Inhaled steroids	Diffuses freely into immune cells where they interact with cytoplasmic glucocorticoid receptors and target TFs such as NF-kB and AP-1 (Saeki et al., 2004)	Decreases expression of CD80 and CD86 on MoDCs but leaves HLA-DR unmodified (Saeki et al., 2004)
Cromones	inhibit the activation of human mucosal mast cells by blocking degranulation of mast cells by IgE (Holgate and Polosa, 2008)	No studies reported on MoDCs or human monocytes
muscarinic receptor antagonist (Ipratropium, short-acting, or Tiotropium, long-acting)	Block Muscarinic acetylcholine receptor on on airway sensory nerves which prevents bronchoconstriction (Holgate and Polosa, 2008)	Tiotropium inhibits TH2 cytokine release in allergen-exposed sensitized mice and that from human PBMCs (Holgate and Polosa, 2008)

Conclusion

The work presented in this thesis investigated the innate immune responses to *C. pneumoniae* infection in MoDCs and how it can exacerbate established asthma in humans. An important aspect of *C. pneumoniae* pathogenesis is its ability to evade the immune system and persist within the host. Data from this study demonstrated that *C. pneumoniae* infection of MoDCs leads to the induction of apoptosis and cell death. This suggests that MoDCs have a microbial response pathway activating apoptosis to limit *C. pneumoniae* replication as a mechanism to prevent replication and dissemination.

Altered function of MoDCs from asthma patients have been hypothesised to be the cause for the development of allergic airway inflammation (Gill, 2012). This study investigated the comparative effect of *C. pneumoniae* infection on MoDC maturation markers from healthy controls, acute asthma and stable asthma cohorts. Results indicated that CD80 marker on MoDCs was significantly down-

regulated in both healthy controls and the asthma patients. This down regulation of CD80 by *C. pneumoniae* potentially prevents the generation of Th1 responses by MoDCs. Meanwhile expression of CD83 marker on MoDCs was significantly up-regulated in the stable asthma cohort only. Hence, a combined down-regulation of CD80 and up-regulation of CD83 is observed on *C. pneumoniae* infected MoDCs from stable asthma patients and whether this contributes to their potent Th2 stimulatory properties has to be further explored using MLR studies.

Immunity against *C. pneumoniae* requires cell-mediated mechanisms that result in the production of cytokines such as IL-12 and IFN-γ. Investigation into the cytokine secretion profile revealed that *C. pneumoniae*-infected MoDCs from both asthma cohorts and healthy controls display a significantly enhanced secretion of IL-4 but no IL-12 secretion which suggests bias towards Th2 responses. Furthermore, *C. pneumoniae*-infected MoDCs from asthma cohorts display significantly increased cytokine producing capacity. This increased cytokine secretion profile can drive asthma exacerbation by mediating contraction of airway smooth muscle cells by IL-1β/TNF-α while IL-8 presence can act as chemoattractan for neutrophils which possibly could lead to neutrophilic asthma. Meanwhile, increased production of IL-4, IL-6 and IL-10 by *C. pneumoniae*-infected MoDCs from asthma cohorts can influence Th2 responses which can lead to asthma exacerbations.

Disequilibrium of innate immune responses and regulatory mechanisms in response to respiratory pathogens results in allergic disorders such as asthma. To provide greater insight into underlying immune responses against *C. pneumoniae*, genome-wide transcriptional profiling was utilised. Results identified specific gene expression profiles in the asthma cohort which includes selective expression of airway remodelling genes and increased expression of inflammatory genes such as chemokines and CSFs. *C. pneumoniae*-infected MoDCs from asthma patients displayed a significantly increased expression of chemokine and CSF genes which acts together to attract inflammatory cells such as eosinophils, neutrophil and MoDCs into the respiratory tract and prolong their survival. This can promote release of inflammatory mediators in the airways triggering airway inflammation. Moreover, selective expression of airway remodelling genes such as ADAMs, MMPs, PAIs and VEGF in the asthma cohort can contribute to *C. pneumoniae*-induced asthma through structural changes in the airway epithelium. Despite the fact that the asthma cohorts in this study were also on medications, *C. pneumoniae* infection can still induce expression of chemokines, CSFs, cytokines and airway remodelling genes. Hence, this raises concern regarding exposure to infectious *C. pneumoniae* in asthma patients.

Future directions

Due to time constraints, we could not undertake further work to validate the microarray findings by quantitative reverse transcription PCR (qRT-PCR). Hence, future work needs to be aimed at validating the gene expression levels of the airway remodelling genes and inflammatory response genes from the asthma cohort by qRT-PCR. Functional validation studies should be carried out to deduce whether DC derived MMPs from asthma patients can permeabilise bronchial epithelial cells *in vitro* to mimic *C. pneumoniae* infection in bronchial epithelium (Marsac et al., 2011). This will help us deduce whether soluble factors derived from *C. pneumoniae*-infected MoDCs can aggravate airway damage in asthma patients. Furthermore, MLR studies needs to be carried out to determine the Th2 driving potential of *C. pneumoniae*-infected MoDCs from the asthma cohort.

The work demonstrated in this thesis can be extended to study the effect of *C. pneumoniae* infection on childhood asthma using a cohort of paediatric asthma patients. Infections early in life are a major and potential long-term risk factor for subsequent wheezing and asthma. Several epidemiologic studies suggest that infants who develop severe viral respiratory infections are more likely to have asthma later in childhood (Holt and Sly, 2012; Holt et al., 2012). Murine studies have shown that early life chlamydial infection can predispose to asthma in later life during adulthood (Horvat et al., 2007; Horvat et al., 2010b) which is possibly mediated through alterations in hematopoietic cells that affects pulmonary immunity, function and structure which might predispose to asthma in later life (Starkey et al., 2012b). It would be interesting to see if similar observation can also be made in paediatric asthma patients as MoDCs isolated from children under 12 year old produce lower amounts of IL-12 in response to microbial stimulation when compared with adults (Upham et al., 2002). This will help us deduce whether *C. pneumoniae* infection in MoDCs from paediatric asthma patients can drive a stronger Th2 response.

The final outcome and application of this study could be used in the development of therapeutics aimed at tuning the magnitude of DC responses in *C. pneumoniae*-induced asthma. Although such questions are beyond the scope of the research presented herein, we surmise that the experimental data that we have presented as part of this thesis work furnish a productive platform of inquiry. We suggest that understanding the basic mechanisms underlying the interactions between *C. pneumoniae* and asthma is necessary to develop primary prevention strategies as well as focused therapeutics in asthma.

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APPENDICES

Supplementary Table 1: List of highly up-regulated genes in healthy controls

Table shows list of genes which were significantly up-regulated in *C. pneumoniae*-infected DCs from healthy controls (n=5) when compared to uninfected DCs. Genes were selected based on criteria of differential regulation of 2-fold ± expression and $p<0.05$. Fold change values represent mean fold changes in *C. pneumoniae*-infected DCs when compared with uninfected DCs.

Supplementary Table 2: List of highly down-regulated genes in healthy controls

Table shows list of genes which were significantly down-regulated in *C. pneumoniae*-infected DCs from healthy controls (n=5) when compared to uninfected DCs. Genes were selected based on criteria of differential regulation of 2-fold ± expression and $p<0.05$. Fold change values represent mean fold changes in *C. pneumoniae*-infected DCs when compared with uninfected DCs.

Supplementary Table 3: List of highly up-regulated genes in acute asthma cohort

Table shows list of genes which were significantly up-regulated in *C. pneumoniae*-infected DCs from acute asthma cohort (n=5) when compared to uninfected DCs. Genes were selected based on criteria of differential regulation of 2-fold ± expression and $p<0.05$. Fold change values represent mean fold changes in *C. pneumoniae*-infected DCs when compared with uninfected DCs.

Supplementary Table 4: List of highly down-regulated genes in acute asthma cohort

Table shows list of genes which were significantly up-regulated in *C. pneumoniae*-infected DCs from acute asthma cohort (n=5) when compared to uninfected DCs. Genes were selected based on criteria of differential regulation of 2-fold ± expression and $p<0.05$. Fold change values represent mean fold changes in *C. pneumoniae*-infected DCs when compared with uninfected DCs.

Supplementary Table 5: List of highly up-regulated genes in stable asthma cohort

Table shows list of genes which were significantly up-regulated in *C. pneumoniae*-infected DCs from stable asthma cohort (n=5) when compared to uninfected DCs. Genes were selected based on criteria of differential regulation of 2-fold ± expression and $p<0.05$. Fold change values represent mean fold changes in *C. pneumoniae*-infected DCs when compared with uninfected DCs.

Supplementary Table 6: List of highly down-regulated genes in stable asthma cohort

Table shows list of genes which were significantly up-regulated in *C. pneumoniae*-infected DCs from stable asthma cohort (n=5) when compared to uninfected DCs. Genes were selected based on criteria of differential regulation of 2-fold ± expression and $p<0.05$. Fold change values represent mean fold changes in *C. pneumoniae*-infected DCs when compared with uninfected DCs.

Supplementary Table 7: Comparison of differentially expressed genes from healthy controls, acute asthma and stable asthma cohort

Table shows list of genes which are differentially expressed in *C. pneumoniae*-infected DCs from healthy controls, acute asthma and stable asthma cohort when compared to uninfected DCs. Genes can only be considered significantly expressed based on criteria of differential regulation of 2-fold ± expression (1 on the $\log_2$ scale) and $p<0.05$. Log ratio values represent gene expression ratios

(expression level in *C. pneumoniae*-infected DCs **divided** by the expression level in uninfected DCs) on the $\log_2$ scale